UNIVERSIDAD AUTONOMA DE MADRID
Facultad de Ciencias
Departamento de Biología Molecular

"RNA silencing suppression, a novel function for P1 serine proteases of viruses of the family *Potyviridae*"

ADRIAN VALLI

Madrid, septiembre de 2010

UNIVERSIDAD AUTONOMA DE MADRID
Facultad de Ciencias
Departamento de Biología Molecular

"RNA silencing suppression, a novel function for P1 serine proteases of viruses of the family *Potyviridae*"

Memoria presentada por Adrian Valli para optar al Título de Doctor

Esta tesis doctoral se ha realizado en el Departamento de Genética Molecular de Plantas del Centro Nacional de Biotecnología (CNB-CSIC) bajo la dirección del Dr. Juan Antonio García

El trabajo presentado en esta memoria ha sido posible gracias al disfrute de una beca pre-doctoral I3P del Consejo Superior de Investigaciones Científicas - CSIC.

Madrid, septiembre de 2010

ABBREVIATIONS

Measure units: International units

3D	Three-dimensional
aa	Amino acid
ATPase	Adenosine tri-phosphate
bp	Base pair
BSA	Bovine serum albumin
cDNA	copy DNA
CI	Cylindrical inclusion
CP	Capsid protein
DCL	DICER-LIKE nuclease
DNA	Deoxyribonucleic acid
dpa	Days post agro-infiltration
dpi	Days post infection
dsRNA	Double-stranded RNA
EDTA	Ethilen-diamin tetraacetic acid
EGTA	Ethilen-glycol tetraacetic acid
EMSA	Electrophoretic mobility shift assay
GFP	Green fluorescent protein
HCPro	Helper component protein
IgG	Immunoglobulin G
kDa	Kilo Daltons
miRNA	Micro RNA
MOPS	3-(N-morpholino)propanesulfonic acid
mRNA	Messenger RNA
NCR	Non-coding region
NI	Nuclear inclusion
nt	Nucleotide(s)
OD	Optical density
ORF	Open reading frame
PCR	Polymerase chain reaction
PIPO	Pretty interesting potyvirus ORF
RDR	RNA-dependent RNA polymerase
rgsCaM	Calmodulin-related protein
RISC	RNA-induced silencing complex
RITS	RNA-induced transcriptional silencing complex
RNA	Ribonucleic acid
RSS	RNA silencing suppressor
RSS	RNA silencing suppressor
SDS	Sodium dodecyl sulphate
SDS-PAGE	Polyacrylamide gel electrophoresis in presence of SDS
siRNA	Small interferent RNA
ssRNA	Single stranded RNA
TBE	Tris-boric acid 90 mM, Na2-EDTA 2 mM pH8 buffer
UV	Ultraviolet
wt	Wild type
YFP	Yellow fluorescent protein

VIRUSES CITED (except those viruses belonging to *Potyviridae* family, which are listed in table M6 of *Materials and Methods*).

ACLSV	*Apple chlorotic leaf spot virus*
ACMV	*African cassava mosaic virus*
ASLV	*Apple latent spherical virus*
BCTV	*Beet curly top virus*
CaMV	*Cauliflower mosaic virus*
EACMCV	*East African cassava mosaic Cameroon virus*
MYMV	*Mungbean yellow mosaic virus*
PolV	*Pothos latent virus*
PVX	*Potato virus X*
RCNMV	*Red clover necrotic mosaic virus*
RNYVV	*Beet necrotic yellow vein virus*
SPCSV	*Sweet potato chlorotic stunt virus*
TGMV	*Tomato golden mosaic virus*
ToCV	*Tomato chlorotic virus*
TuYV	*Turnip yellow virus*
TYLCV	*Tomato yellow leaf curl virus*
TSWV	*Tomato spot wilt virus*
CIRV	*Carnation Italian ringspot virus*

Resumen

Introducción.

Silenciamiento de RNA, defensa frente a infecciones virales y supresores de silenciamiento.

El término silenciamiento de RNA, de manera colectiva, hace referencia a diversas vías que controlan, mediante regulación de la expresión génica, una gran variedad de procesos fisiológicos, tales como patrones de desarrollo, respuestas a condiciones de estrés, mantenimiento de la estabilidad génica y defensa frente a ácidos nucleicos invasores, en animales, plantas y hongos. Estos sistemas de regulación se activan por moléculas de RNA de banda doble, total o parcialmente complementarias, que son procesadas por enzimas de tipo RNAsa III pertenecientes a la familia Dicer para producir pequeños (aproximadamente 21-24 nt) RNAs bicatenarios con 2 nt colgantes en sus extremos 3'. Estas pequeñas moléculas se asocian luego con complejos efectores que contienen una proteína de la familia Argonaute, para guiarlos, por complementariedad de bases, hacia los genes diana (Baulcombe, 2005). Estos complejos ribonucleoproteicos pueden producir silenciamiento post-transcripcional, mediado por un corte específico, desestabilización o represión de la traducción de los RNAs diana, o inducir cambios epigenéticos y remodelación de la cromatina inhibiendo así la transcripción (Ghildiyal & Zamore, 2009; Vaucheret, 2008; Verdel *et al.*, 2004).

Existen varios tipos de RNAs pequeños, los cuales se clasifican según su biogénesis. En plantas, por ejemplo, existen principalmente dos clases: microRNAs y RNAs pequeños interferentes [***s**mall **i**nterfering* (si)RNAs](Chen, 2009). Nuestro conocimiento acerca del silenciamiento de RNA en plantas deriva ampliamente de los análisis realizados en la planta modelo *A. thaliana*. En este organismo, la maquinaria de silenciamiento está formada por: i) cuatro proteínas de tipo Dicer, ii) diez proteínas de la familia Argonaute y iii) seis RNA polimerasas dependientes de RNA, que están implicadas en la amplifican del sistema. Trabajos realizados con este organismo muestran cómo las diversas vías de silenciamiento de RNA están formadas por diferentes combinaciones de módulos que incluyen un número limitado de componentes que actúan de manera parcialmente redundante constituyendo redes complejas altamente reguladas que actúan sobre el mRNA o la cromatina.

Está bien establecido que el silenciamiento de RNA actúa en plantas como respuesta inmune frente a infecciones virales (Mlotshwa *et al.*, 2008b). Tanto en la infección de virus con genoma de DNA como en la de aquellos con genoma de RNA

la maquinaria de silenciamiento del huésped produce siRNAs virales que dirige a los complejos efectores para degradar a los ácidos nucleicos invasores. Por su parte, los virus han tenido que evolucionar para poder escapar de esta barrera defensiva y ser capaces de establecer una infección. En el año 1998 tres grupos de manera independiente descubrieron que la proteína potyviral HCPro suprimía el silenciamiento de RNA (Anandalakshmi *et al.*, 1998; Brigneti *et al.*, 1998; Kasschau & Carrington, 1998). Diferentes aproximaciones se han utilizado desde ese momento para identificar un gran número de factores virales con la misma capacidad que HCPro, indicando que la expresión de proteínas con actividad supresora de silenciamiento es una estrategia común empleada por los virus para evadir esta respuesta inmune de las plantas.

Los supresores de silenciamiento no tienen secuencias de aminoácidos conservadas ni evidentes similitudes estructurales. Esta clara divergencia sugiere que han surgido en eventos evolutivos recientes e independientes. Sólo se han llevado a cabo estudios detallados de los mecanismos moleculares de supresión de silenciamiento para unas pocas proteínas supresoras, pero todo apunta a que estos factores virales tienen mecanismos de acción muy específicos.

La familia Potyviridae *de virus de plantas:*

La familia *Potyviridae* es probablemente el grupo más grande de virus de plantas (Berger *et al.*, 2005). Incluye al género *Potyvirus* con sus más de cien miembros definidos (y con el mismo numero de miembros tentativos), caracterizados por ser transmitidos por pulgones. Los otros virus con genoma monopartido de la familia están distribuidos en los géneros *Macluravirus*, también transmitidos por pulgones, *Ipomovirus,* transmitidos por mosca blanca, y *Rymovirus* y *Tritimovirus,* transmitidos por ácaros. Los miembros del género *Bymovirus*, el restante género de la familia, tienen genoma bipartito y se transmiten por plasmodióforos. Virtualmente, cualquier cultivo de uso agronómico puede sufrir pérdidas causadas por uno o más miembros de esta gran grupo viral, rasgo que le da a la familia *Potyviridae* un gran interés socioeconómico.

Los virus de la familia *Potyviridae* tienen un genoma de RNA de cadena sencilla y polaridad positiva con un tamaño que va, para los miembros monopartidos, desde 8.2 hasta 11 kb. El RNA genómico tiene una cola de poli A en su extremo 3', y a diferencia de los mRNAs celulares, presenta una proteína viral unida

covalentemente a su extremo 5' (VPg) en lugar de una estructura "cap" (Riechmann *et al.*, 1992). El genoma de los potyvirus se encapsida en viriones helicoidales flexibles de una longitud de entre 680 y 900 nm y una anchura entre 11 y 15 nm, que están formados por unas 2.000 unidades de un único tipo de proteína estructural (CP) (Shukla *et al.*, 1994).

El RNA genómico de los potyvirus tiene un marco de lectura abierto que se traduce en una poliproteína de gran tamaño, que se procesa proteolíticamente por proteasas vírales. Así pues, los potyvirus son similares en cuanto a estructura genómica y estrategia de expresión al resto de miembros del súper grupo de virus tipo Picorna. En la mayoría de los miembros monopartidos de la familia, los siguientes productos forman la poliproteína viral (desde el N- hasta el C-terminal): P1, HCPro, P3, 6K1, CI, 6K2, NIa(VPg+Pro), NIb y CP. Adicionalmente, un producto viral llamado P3+PIPO, embebido en la región de P3 y generado por un cambio en el marco de lectura, es producido. De manera sorpresiva, se han hallado recientemente excepciones en la estructura genómica de algunos virus aún incluidos en el género *Ipomovirus*, los cuales carecen en su genoma de la región que codifica a la proteína HCPro (Janssen *et al.*, 2005; Li *et al.*, 2008; Mbanzibwa *et al.*, 2009).

Inicialmente, HCPro fue considerada la única proteína de los virus de la familia *Potyviridae* con actividad supresora del silenciamiento, por lo que se asumió que era esencial para todos ellos. La primera evidencia de la existencia de la divergencia genética marcada por la ausencia de HCPro se descubrió al analizar la secuencia del genoma de CVYV, en donde además se observó la existencia de un cistrón extremadamente largo para la proteína P1 (Janssen *et al.*, 2005). Con estos antecedentes, el objetivo general de este trabajo de tesis doctoral fue identificar y caracterizar las proteínas virales codificadas en el extremo 5' de los genomas virales de diferentes potyvirus, y determinar sus contribuciones a la supresión de la respuesta defensiva de la planta basada en silenciamiento de RNA. Yo estaba especialmente interesado en entender cómo diferentes virus tan íntimamente relacionados dependían de proteínas tan diversas para contraatacar a la planta.

Resultados:

Eventos de recombinación y duplicación génica en la diversificación evolutiva de las proteínas P1 en la familia Potyviridae.

A pesar de la notable conservación en la estructura y secuencia del genoma de los virus de la familia *Potyviridae*, algunas regiones de estos virus, especialmente la que codifica a la proteína P1, son altamente variables. Así, uno de los objetivos puntuales de esta tesis fue inferir las posibles funciones de las proteínas P1 en el ciclo infectivo a partir de un análisis de secuencia usando aproximaciones *in silico*. Mediante el uso de herramientas informáticas, se encontraron diversos motivos parcialmente conservados, además del dominio serín proteasa, a lo largo de toda la proteína, los cuales mostraban a su vez una distribución muy irregular, sugiriendo que el cistrón correspondiente a P1 ha evolucionado de manera muy compleja.

También se investigó la presencia de recombinaciones. Para ello se seleccionaron ejemplos en los cuales estos eventos se podían inferir a partir de la secuencia de las proteínas implicadas. Se encontraron así evidencias de recombinaciones en la región N-terminal de varias P1s, que eran similares a las reportadas previamente para los miembros del sub-grupo de BCMV (Desbiez & Lecoq, 2004; Larsen *et al.*, 2005). De la misma manera también fuimos capaces de detectar recombinaciones entre miembros de diferentes géneros, así como también evidencias de que estos eventos podrían estar contribuyendo a la adaptación a diferentes huéspedes.

El análisis de ciertas características en las secuencias de las P1s y sus puntos isoeléctricos permitieron clasificar a estas proteínas en dos grupos bien definidos: P1s “clásicas” y proteínas “tipo tritimo”. Sorpresivamente se pudo ver *in silico* que el ipomovirus CVYV tenía dos copias seguidas de P1 en su genoma, una del tipo clásico y la otra del “tipo tritimo”. Estas proteínas fueron bautizadas con el nombre de P1a y P1b, por lo que luego se decidió rebautizar al segundo grupo de proteínas P1, denominándolas “tipo P1b”.

Búsqueda y caracterización del supresor de silenciamiento de CVYV.

El cistrón de HCPro estaba presente en todos los miembros monopartidos conocidos de la familia, hasta que se secuenció en el año 2005 el genoma del ipomovirus CVYV y se encontró que este cistrón estaba ausente (Janssen *et al.*, 2005). Utilizando técnicas rutinarias se consiguió amplificar y clonar, a partir de hojas

de pepino infectadas, la secuencia del extremo 5' de CVYV que incluía la región 5' no codificante y el cistrón de la hipotética proteína P1. Mediante la expresión transitoria en plantas por agroinfiltración pudimos expresar esta proteína marcada y determinar que, tal como habíamos predicho mediante los estudios *in silico*, esta gran proteína se autoprocesa muy eficientemente para dar lugar a dos serín proteasas homólogas: P1a y P1b. La observación de la acumulación de P1b procesada en una infección natural de CVYV confirmó definitivamente nuestra hipótesis de dos P1s en *tandem*.

Dada la ausencia de HCPro, el típico supresor de silenciamiento potyviral, se pensó que algunas de las P1s de CVYV podía suplir su actividad. Mediante diferentes análisis de co-agroinfiltración usando el RNA mensajero de la GFP como reportero se pudo ver que P1b, pero no P1a, era capaz de inhibir la degradación por silenciamiento del RNA reportero. Toda la batería de análisis realizados mostraron que P1b de CVYV y HCPro de PPV suprimían el silenciamiento de RNA de manera parecida.

Dominios conservados en proteínas "tipo P1b". Relevancia de los mismos en diferentes actividades de P1b de CVYV.

Un alineamiento de la secuencia de aminoácidos de las proteínas "tipo P1b" reveló que, además de la región C-terminal conservada correspondiente al dominio serín proteasa, existían otros dos dominios conservados que se localizaban en la mitad N-terminal de estas proteínas: un posible dedo de zinc y un motivo con la firma LxKA.

Mediante expresión transitoria en planta de una construcción reportera, tanto de una versión silvestre como de mutantes puntuales del posible centro activo proteolítico, se comprobó que P1b es una típica serín proteasa que se autoprocesa en el extremo carboxilo. También utilizando mutagénesis dirigida, se ha determinado la gran relevancia en la supresión de silenciamiento del motivo LxKA y del dedo de zinc, observando además que la actividad proteasa no es necesaria para suprimir el silenciamiento de RNA. Estudios adicionales de filtración en gel/FPLC *in vitro* y análisis de complementación bimolecular *in vivo*, mostraron que P1b interacciona consigo mismo formando oligómeros, posiblemente homodímeros. Estos análisis han puesto de manifiesto también el rol esencial que juega el dedo de zinc en la formación de estas estructuras.

Determinación del mecanismo molecular de la supresión de silenciamiento mediada por P1b.

Se había determinado que el supresor HCPro de TEV suprime el silenciamiento mediante interacción directa con los siRNAs (Lakatos *et al.*, 2006). Dada la gran similitud funcional encontrada entre P1b de CVYV y HCPro de PPV en todos los ensayos que se habían realizado, se decidió analizar si P1b también poseía esta capacidad, obteniéndose resultados positivos. Los datos obtenidos mediante ensayos de salto de movilidad electroforética han revelado que P1b interacciona sólo con siRNAs de banda doble mostrando una preferencia por aquellos de 21 nt de longitud. Es interesante que, a diferencia de lo observado para HCPro, el reconocimiento de los siRNAs por parte de P1b no está influenciado por la presencia de los 2 nt colgantes en el extremo 3' de los siRNAs, ni por el grupo fosfato presente en el extremo 5' de los mismos. Esta capacidad de P1b se ha podido confirmar *in vivo* mediante ensayos de co-purificación. Además, un análisis de secuenciación masiva de las poblaciones de RNA que interaccionaban con P1b *in vivo* reveló que la preferencia de tamaño por siRNAs de 21 nt ocurre también en la planta, y que la expresión de P1b produce acusados cambios en los patrones de los RNAs pequeños endógenos. Por último, mediante un análisis de mutagénesis dirigida se encontró que el dominio conservado básico LxKA media la interacción de P1b con los siRNAs.

Dada la inexistencia de un clon infectivo de cDNA de CVYV, la relevancia de P1b en el contexto de una infección viral se analizó en un sistema homólogo basado en PPV. Así, estudiando en detalle los procesos infectivos de virus quiméricos, se demostró que P1b de CVYV es capaz de reemplazar a HCPro de PPV, y que esta capacidad se correlacionaba perfectamente con la capacidad de P1b de unir siRNAs y suprimir el silenciamiento de RNA.

Todos los resultados obtenidos nos sugieren muy fuertemente que P1b interfiere con el silenciamiento de RNA mediante el secuestro de los siRNAs, impidiendo la degradación viral, quizás, previniendo la incorporación de estas moléculas en los complejos efectores.

Discusión

Nuestro entendimiento de la evolución de los virus de plantas está creciendo día tras día como consecuencia del renovado interés en el campo, el cual ha sido causado, en parte, por lo útil que resultan los virus de plantas como sistemas modelos. Los datos descritos en esta tesis ilustran, por ejemplo, la extensa divergencia de la región correspondiente a la proteína P1 de los miembros de la familia *Potyviridae*. Basándonos en características simples de sus secuencias de aminoácidos se puede agrupar a estas proteínas en dos tipos: "clásicas" y "tipo P1b".

Los datos mostrados reflejan también una gran divergencia dentro del grupo de las P1s clásicas, y dan claras evidencias de que la recombinación de RNA ha jugado un papel crucial en la diversificación de estas proteínas, habiéndose encontrado eventos de este tipo inclusive entre virus de la familia pertenecientes a géneros distintos. Todos estos datos sugieren que la recombinación cumple un rol importante en la adaptación de estos virus a diferentes huéspedes. Además, como la mayoría de las recombinaciones que se han descrito afectan a la mitad N-terminal de las proteínas P1, cabe pensar que esta región pueda estar involucrada en interacciones específicas con factores de la planta que contribuyen a esta adaptación.

Los análisis *in silico* mostraban la existencia de dos serín proteasas en la región N-terminal de la poliproteína de CVYV, sugiriendo que lo que se propuso originalmente como una P1 extremadamente grande eran en realidad dos proteínas P1 independientes: P1a, perteneciente al grupo de las P1 clásicas y P1b, prototipo del grupo "tipo P1b". Estas predicciones se confirmaron *in vivo* mediante diferentes aproximaciones experimentales, incluido el análisis concluyente de la acumulación de la proteína P1b procesada en una infección natural de CVYV en plantas de pepino. De esta manera, pese a que CVYV carece de la cisteín proteasa HCPro, su estrategia de expresión génica es muy similar a la utilizada por la mayoría de los virus de la familia *Potyviridae,* la cual supone el procesamiento de la poliproteína viral mediante la acción de tres proteasas codificadas por el propio virus.

La ausencia de la proteína HCPro en el genoma de ciertos ipomovirus originaba muchas preguntas acerca de cómo estos virus pueden suplir la falta de las actividades suministradas por HCPro, especialmente aquella necesaria para suprimir el silenciamiento de RNA. Los experimentos realizados en esta tesis demuestran que la proteína P1b de CVYV es capaz de suprimir el silenciamiento en diversos

sistemas experimentales, por lo que este factor estaría reemplazando a HCPro en el genoma de CVYV, al menos en esta función. Estos resultados, junto con otros reportados recientemente, indican que la capacidad de suprimir el silenciamiento de RNA es probablemente una característica general de las proteínas P1 "tipo P1b" (Giner *et al.*, 2010; Mbanzibwa *et al.*, 2009; Stenger, 2007), que las diferencia de las P1 clásicas, aunque los mecanismos empleados pueden variar entre diferentes proteínas "tipo P1b", como parece ser el caso de P1b de CVYV (datos de esta tesis) y P1 de SPMMV (Giner *et al.*, 2010).

La interacción con siRNAs es una estrategia utilizada por diferentes factores virales para suprimir el silenciamiento. Por ejemplo, la proteína HCPro de los potyvirus y la proteína P19 de los tombusvirus muestran una alta preferencia por unir siRNAs de 21nt, inhibiendo así su incorporación en los complejos efectores (Lakatos *et al.*, 2006). Mediante análisis *in vitro* e *in vivo* se observó que P1b comparte con estos supresores de silenciamiento no sólo la capacidad de unión siRNAs, sino también la misma preferencia de tamaño. Sin embargo, basándonos en los datos *in vitro* aquí obtenidos, y en otros previamente reportados (Lakatos *et al.*, 2006; Vargason *et al.*, 2003), respecto a las preferencias de los supresores hacia ciertas características presentes en los extremos de los siRNAs, se puede concluir que la unión desplegada por P1b es diferente a las que exhiben P19 y HCPro. Esto, junto con la falta de similitud en la secuencia proteica de los tres supresores, pone de manifiesto cómo estrategias funcionales similares se pueden conseguir mediante líneas evolutivas independientes.

Pese a que la región N-terminal de las proteínas P1 "tipo P1b" está pobremente conservada, es posible reconocer dos motivos parcialmente conservados. El primero de ellos, denominado LxKA, no muestra coincidencias con dominios previamente anotados en las bases de datos, mientras que el segundo fue identificado como un dedo de zinc de tipo $Cx_2Cx_nCx_2C$. Ambos motivos tienen una gran relevancia en las funciones de P1b, ya que mutaciones específicas en los mismos reducen drásticamente la actividad supresora de silenciamiento. Adicionalmente, estudios detallados *in vitro* e *in vivo* de la conformación de versiones silvestres y mutantes de P1b indican que este factor interacciona consigo mismo dando lugar a oligómeros, probablemente homodímeros, en cuya formación el dedo de zinc está involucrado. Por su parte, el motivo conservado LxKA era un buen candidato a estar involucrado en la interacción con los siRNAs debido a que ésta región es rica en aminoácidos

básicos, los cuales a menudo participan en interacciones supresor-siRNA. Análisis *in vitro* e *in vivo* realizados con mutantes específicos confirmaron que el motivo LxKA está involucrado en la unión de los siRNAs. Además, la precisa correlación encontrada entre la unión de los siRNAs *in vivo* y la actividad supresora del silenciamiento de los diferentes mutantes de P1b apoyan fuertemente la conclusión de que este supresor bloquea el silenciamiento de RNA interfiriendo específicamente con una función de los siRNAs.

El estudio llevado a cabo con virus quiméricos de PPV no solo muestra que P1b es capaz de reemplazar a HCPro en una infección potyviral y que PPV no depende de funciones exclusivas de HCPro durante el proceso infeccioso, sino también que, a diferencia de lo que ocurre con diversos virus de otras familias (Diaz-Pendon *et al.*, 2007; Omarov *et al.*, 2006; Omarov *et al.*, 2007; Qiu *et al.*, 2002; Soards *et al.*, 2002; Szittya *et al.*, 2002; Ziebell & Carr, 2009; Ziebell *et al.*, 2007), los potyvirus no son capaces de iniciar una infección en ausencia de un supresor de silenciamiento activo. Todos estos resultados demuestran el papel clave de los supresores de silenciamiento para la viabilidad viral.

Quedan muchas cuestiones interesantes para resolver en el futuro, por ejemplo, entender cómo proteínas tan diferentes (P1b de CVYV, HCPro, P19) tienen una actividad supresora de silenciamiento tan similar, y cómo proteínas íntimamente relacionadas (P1b de CVYV y P1 de SPMMV, ambas “tipo P1b”) han adoptado mecanismos tan diferentes para neutralizar el mismo sistema antiviral hacia virus tan parecidos. Más aún, si las proteína P1 “tipo P1b” tienen roles adicionales independientes de la supresión de silenciamiento, tal como los tiene HCPro, se nos plantea el desafío de estudiar las relaciones estructura/función implicadas en estas actividades, con objeto no sólo de entender las funciones de estas fascinantes proteínas, sino también la compleja evolución de los virus de la familia *Potyviridae*.

Introduction

I. INTRODUCTION

I.1 RNA silencing in plants and its suppressors*[1]

I.1.1 General considerations

RNA silencing refers collectively to the diverse regulatory pathways that control gene expression in eukaryotic organisms and involve small RNAs, which are mainly derived from the RNase III–like–mediated processing of dsRNAs (Baulcombe, 2005; Susi *et al.*, 2004). An RNA silencing pathway underlies the co-suppression phenomenon described in transgenic petunias in 1990. This co-suppression was characterized by a reduction in the steady-state mRNA level of endogenous genes, of which extra copies had been added to the nuclear genome (Napoli *et al.*, 1990; van der Krol *et al.*, 1990). The same mechanism was shown to be involved in the specific constitutive or delayed antiviral resistance of plants transformed with virus-derived transgenes (English *et al.*, 1996; Lindbo *et al.*, 1993). The role of RNA silencing was later acknowledged to be a general plant defense mechanism against viral infections (Covey *et al.*, 1997; Ratcliff *et al.*, 1997). This was further supported by the discovery of viral proteins, termed RNA silencing suppressors (RSSs), able to block or interfere with this mechanism (Anandalakshmi *et al.*, 1998; Béclin *et al.*, 1998; Brigneti *et al.*, 1998; Kasschau & Carrington, 1998).

Many recent studies have revealed that RNA silencing is a very complex, homology-dependent gene expression regulatory system composed of different modules that use the combinatorial, hierarchical, and partially redundant actions of shared components. Furthermore, RNA silencing is present not only in plants, but also in a wide range of eukaryotic organisms, including fungi, worms, insects, and vertebrates (Dunoyer & Voinnet, 2008; Smith *et al.*, 2007; Susi *et al.*, 2004).

I.1.2 Molecular mechanism of RNA silencing: Core steps

RNA silencing operates through several pathways with a core machinery (Fig. I1) (Vaucheret, 2006). This regulatory system is activated by either perfectly- or partially-paired dsRNA molecules, which are processed by RNase III-like enzymes in the Dicer family to produce short (approximately 21-24 nt) RNA duplexes with 2-nt overhangs at their 3' ends. Although some species, such as *Caenorhabditis elegans* and humans, contain a single Dicer,

*[1] This section is an update of **Valli, A., López-Moya, J. J. & García, J. A. (2009).** RNA silencing and its suppressors in the plant-virus interplay. In *Encyclopedia of Life Sciences (ELS)*, p. http://www.els.net/ [DOI: 10.1002/9780470015902.a9780470021261]. Chichester: John Wiley & Sons, Ltd.

several Dicer-like genes are found in other organisms, allowing more sophisticated RNA silencing pathways to be developed (Chapman & Carrington, 2007). Thus, *Arabidopsis thaliana* has four DICER-LIKE nucleases (DCL1-DCL4), which produce small RNAs of different sizes: 21 nt (DCL1 and DCL4), 22-24 nt (DCL2) or 24 nt (DCL3). These different DCL proteins have specialized functions; for instance, DCL1 cleaves RNA hairpins yielding miRNAs (Kurihara & Watanabe, 2004; Park *et al.*, 2002; Reinhart *et al.*, 2002), DCL2 contributes to the generation of natural antisense (nat-) short interfering (si) RNAs from complementary overlapping mRNA transcripts (Borsani *et al.*, 2005), DCL3 produces cis-acting siRNAs involved in epigenetic modifications at DNA repeats and transposon loci (ra-siRNAs) (Xie *et al.*, 2004), and DCL4 is responsible for the biogenesis of endogenous trans-acting siRNAs (ta-siRNAs) (Gasciolli *et al.*, 2005; Xie *et al.*, 2005; Yoshikawa *et al.*, 2005), viral siRNAs (Bouché *et al.*, 2006; Deleris *et al.*, 2006), and siRNAs derived from inverted repeat (IR) transgenes (Dunoyer *et al.*, 2005).

The DCL proteins are also partially redundant, and several of them act in a hierarchical manner on shared substrates. For instance, although most miRNAs are produced by DCL1, the accumulation of certain ones appears to depend on DCL4 (Rajagopalan *et al.*, 2006). DCL2 processes viral siRNAs when DCL4 is inactivated (Deleris *et al.*, 2006). Under conditions of extreme dosage, dsRNAs derived from IR transgenes are processed not only by DCL4 cleavage, but also by alternative DCL3-, DCL2-, and DCL1-dependent pathways (Dunoyer *et al.*, 2007). The activity of Dicer and DCL proteins is assisted by companion proteins, such as the *Arabidopsis* dsRNA-binding proteins HYL1 and DRB4, which cooperate with DCL1 and DCL4, respectively (Curtin *et al.*, 2008; Dong *et al.*, 2008; Han *et al.*, 2004; Hiraguri *et al.*, 2005; Vazquez *et al.*, 2004), or the zinc finger protein SERRATE (SE) (Lobbes *et al.*, 2006; Yang *et al.*, 2006) and the FHA protein DAWDLE (DDL) (Yu *et al.*, 2008), which also contribute to DCL1 cleavage activity.

While Dicer and DCL cleavage produce small RNAs of 21-24 nt, poorly characterized small RNAs of longer sizes have also been identified in different organisms. The accumulation of small RNAs of 30-40 nt in *Arabidopsis* following pathogen infection or under specific growth conditions seems to depend on DCL1 and DCL4 (Katiyar-Agarwal *et al.*, 2007). In contrast, Piwi-interacting RNAs (piRNAs), which accumulate in the germline cells of mammals and insects, are Dicer-independent. These piRNAs are derived from the cleavage of RNA precursors by complexes that contain Argonaute proteins and are guided by pre-existing complementary piRNAs (Brennecke *et al.*, 2007; Gunawardane *et al.*, 2007). More recently, a processing pathway independent of Dicer that requires the catalytic activity

of an argonaute protein has been shown to be involved in the generation of some miRNAs of mammals and other organisms (Bosse & Simard, 2010). A variety of small RNAs that do not appear to depend on Dicer for their biosynthesis have also been identified in *C. elegans* (Ruby *et al.*, 2006).

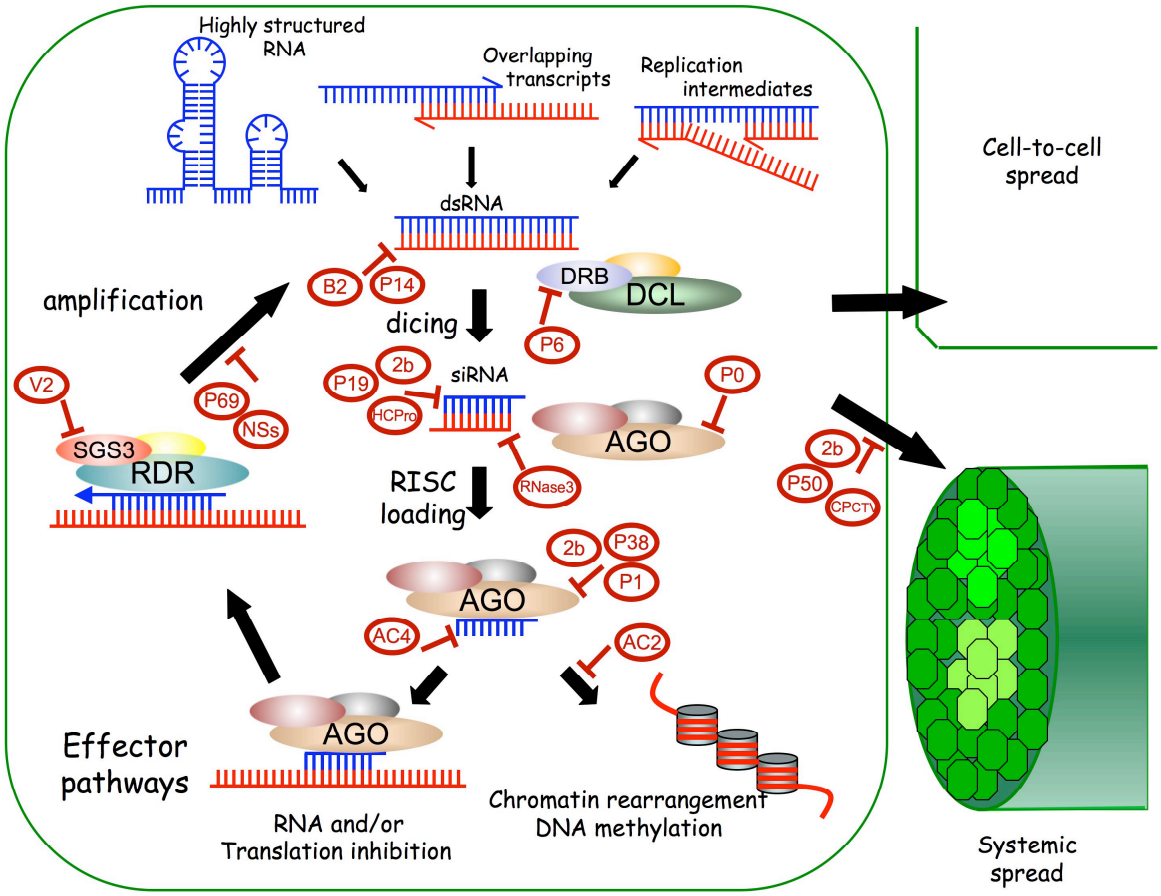


Figure I1. Schematic representation of key steps of plant virus-related RNA silencing. The silencing components that are targeted by some RSSs (depicted as red ovals with their abbreviations inside) are also indicated. See text for details.

The second core step of RNA silencing is carried out by effector complexes, known as RNA-induced silencing complexes (RISCs). These complexes include a protein of the large Argonaute family and a small RNA guide strand (Tolia & Joshua-Tor, 2007). The RISCs can trigger post-transcriptional silencing by specific cleavage (slicing), decay, or translational repression of their mRNA targets; however, in some particular circumstances they can also enhance translational efficiency (Vasudevan *et al.*, 2007). An Argonaute small RNA complex, usually designated as RNA-induced transcriptional silencing complex (RITS), can also induce epigenetic changes and chromatin remodelling, resulting in transcriptional inhibition (Verdel *et al.*, 2004; Zilberman *et al.*, 2003).

It has long been assumed that while translational repression and mRNA destabilization

are the predominant post-transcriptional RNA silencing activities in animals, slicing is the main mode of small RNA–mediated post-transcriptional regulation in plants, and the complementarity between a small RNA and its target sequence principally determines which of these mechanisms is used (Hutvágner & Zamore, 2002). However, it was recently demonstrated that small RNA–mediated translational repression is widespread in plants, and is active even when a high level of complementarity with the guide RNA facilitates the cleavage of the target RNA (Brodersen *et al.*, 2008).

Experiments using *Drosophila* extracts have shown that loading of guide RNA in RISC is promoted by a complex that includes Dicer and a dsRNA-binding protein partner (Liu *et al.*, 2003). However, some Dicers promote RISC assembly without the aid of dsRNA-binding proteins (Liu *et al.*, 2007). The sorting of different small RNAs into specific Argonaute-containing effector complexes appears to depend on the size and structural features of the RNA duplex that are recognized by the corresponding loading complex (Förstemann *et al.*, 2007; Schwarz *et al.*, 2003; Steiner *et al.*, 2007). In addition, the loading complex senses thermodynamic differences between the two ends and chooses the strand that will be incorporated into the mature RISC (Tomari *et al.*, 2007; Tomari *et al.*, 2004). However, the preference of the Argonaute components for particular 5'-terminal nucleotides also contributes to their sorting of small RNAs and to the specific recruitment of one of their strands into RISC complexes (Frank *et al.*, 2010; Mi *et al.*, 2008; Montgomery *et al.*, 2008; Takeda *et al.*, 2008). Strand separation of the small RNA duplex and assembly of the guide strand in the final, active RISC complex can be facilitated by Argonaute-mediated cleavage of the passenger strand (Leuschner *et al.*, 2006; Matranga *et al.*, 2005; Miyoshi *et al.*, 2005; Rand *et al.*, 2005). However, an additional "bypass" mechanism involving RNA duplex unwinding by an unidentified helicase followed by the specific transfer of the guide strand to the Argonaute-containing complex has also been proposed (Preall & Sontheimer, 2005).

The number of Argonaute family members present in some organisms has proliferated to a great extent (Farazi *et al.*, 2008; Höck & Meister, 2008; Tolia & Joshua-Tor, 2007). Although some of these Argonaute proteins may be partially redundant, the expansion of the Argonaute family has probably contributed significantly to the high diversification and specialization of RNA silencing pathways. The number of Argonaute proteins in different organisms is largely variable; for instance, there is one such protein in *Schizosaccharomyces pombe,* five in *Drosophila*, eight in humans, ten in *Arabidopsis*, and 27 in *C. elegans* (Höck & Meister, 2008). Initially, Argonautes were divided into AGO-like and Piwi-like subfamilies, based on their similarity to the AGO1 protein of *Arabidopsis* and the Piwi protein of

Drosophila. However, a group of *C. elegans* Argonaute proteins is distinct from these subfamilies and forms a third clade termed group 3 (Tolia & Joshua-Tor, 2007).

Some Argonaute proteins, like human AGO2 (Liu *et al.*, 2004; Meister *et al.*, 2004), *Drosophila* AGO2 (Rand *et al.*, 2004) and AGO1 (Miyoshi *et al.*, 2005), and *Arabidopsis* AGO1 (Baumberger & Baulcombe, 2005) and AGO4 (Qi *et al.*, 2006) have an RNase H–like motif and display the slicer activity involved in small RNA–guided mRNA cleavage. Piwi subfamily proteins, including *Drosophila* Piwi Aub and AGO3, also have slicer activity (Gunawardane *et al.*, 2007; Saito *et al.*, 2006); the nuclease activity of these two proteins seemingly mediates not only effector function, but also the biosynthesis of piRNAs and some miRNAs, as mentioned above. The RNase H signature is not conserved in all Argonaute proteins, and some that contain an intact catalytic motif still seem to lack slicer activity (Höck & Meister, 2008).

Some specific interactions of Argonautes with other proteins that could be involved in the non-catalytic activities of RNA silencing effector complexes have also been identified. For instance, a PIWI domain pocket interacts with a WG/GW-rich domain present in proteins involved in translational repression and mRNA decay (i.e., GW182) or transcriptional silencing (i.e., Tas3 or NRPD1b) (El-Shami *et al.*, 2007; Till *et al.*, 2007). A cap binding-like motif of AGO proteins could also be relevant for their translational regulatory function (Kiriakidou *et al.*, 2007). Effector roles of some Argonautes might be derived from a combination of several activities. Such appears to be the case for *Arabidopsis* AGO4, which reportedly has distinct catalytic and non-catalytic functions (Qi *et al.*, 2006).

All 10 of the *Arabidopsis* Argonautes are from the AGO family and contain the RNAse H signature. AGO1 is responsible for the slicer activity involved in several forms of post-transcriptional RNA silencing (Baumberger & Baulcombe, 2005; Vaucheret *et al.*, 2004), and may also contribute to the translational repression of a series of miRNA targets (Brodersen *et al.*, 2008). AGO10, the closest paralog of AGO1, is also involved in miRNA-mediated translational repression, although it appears to affect a different subset of miRNA targets than AGO 1 (Brodersen *et al.*, 2008). AGO5 belongs to the same clade as AGO1 and AGO10, but binds siRNAs with a different specificity than AGO1, and its function is presently unknown (Mi *et al.*, 2008; Takeda *et al.*, 2008). AGO4 and AGO6 fall into the same clade and play partially redundant roles in controlling epigenetic changes of chromatin involved in transcriptional gene silencing at specific genomic loci (Zheng *et al.*, 2007; Zilberman *et al.*, 2003). AGO8 and AGO9 are the closest relatives to AGO4 (Tolia & Joshua-Tor, 2007; Vaucheret, 2008), but no data are available about their possible functions. AGO7 can load

miR390 and can function in distinct cleavage/non-cleavage modes at two target sites in a ta-siRNA precursor (Montgomery *et al.*, 2008). AGO2 also loads miR390, but although closely related to AGO7, AGO2 is unable to replace AGO7 in the formation of a synthetic ta-siRNA (Montgomery *et al.*, 2008). The functions of AGO2 and its closest relative AGO3 are still unidentified.

The various RNA silencing pathways are assembled around the two core steps, small RNA synthesis and Argonaute-mediated effector action. A large number of additional partners are involved in the more specific steps, such as the production of the small RNA precursors, small RNA stabilization, and transitive amplification and non–cell autonomous propagation of silencing. Detailed descriptions of these specific steps can be found in some excellent recent reviews (Chapman & Carrington, 2007; Dunoyer & Voinnet, 2008; Kalantidis *et al.*, 2008; Schwab & Voinnet, 2010).

I.1.3 RNA silencing as an antiviral mechanism in plants

More than a decade ago, William Dougherty and his group demonstrated that the transgenic expression of viral RNA sequences could cause a highly specific antiviral state. Although sometimes constitutive, in other instances this antiviral state was induced during recovery from an initial viral infection (Dougherty & Parks, 1995). It was later demonstrated that nuclear transgene silencing establishes viral resistance (English *et al.*, 1996). RNA-mediated antiviral defense also takes place in response to viral infection in the absence of a virus-related transgene; this defense mechanism was responsible for the recovery phenotype observed long time ago in some natural viral infections (Covey *et al.*, 1997; Ratcliff *et al.*, 1997). Today, it is well established that RNA silencing acts as an immune response against viruses, at least in plants and invertebrates (Carrington *et al.*, 2001; Ding & Voinnet, 2007; MacDiarmid, 2005 Mlotshwa, 2008 #6703; Voinnet, 2001).

RNA and DNA viruses produce virus-specific siRNAs when they infect plant cells. Geminivirus siRNAs can be derived from RNA duplexes resulting from the bidirectional transcription of their genomic DNAs (Chellappan *et al.*, 2004). In contrast, a large percentage of siRNAs from DNA virus CaMV are derived from the highly structured leader of its 35S RNA (Moissiard & Voinnet, 2006). Similar amounts of sense and antisense viral siRNAs accumulate in plants infected with several plus-strand RNA viruses. This observation suggests that dsRNA replication intermediates or dsRNA molecules derived from the copy of viral RNAs by host RDRs could be the major source of siRNAs for these viruses (Hamilton & Baulcombe, 1999; Ho *et al.*, 2007; Mlotshwa *et al.*, 2008b). However, the detection of a

disproportionately higher amount of plus-strand siRNAs from specific genomic regions in some viral infections suggests that imperfectly-paired intramolecular duplexes can also be a viral siRNA source (Donaire *et al.*, 2008; Du *et al.*, 2007; Ho *et al.*, 2006; Kurihara *et al.*, 2007; Molnár *et al.*, 2005). Consistent with this suggestion, siRNA strand asymmetry usually (Du *et al.*, 2007; Molnár *et al.*, 2005), but not always (Donaire *et al.*, 2008), correlates with highly folded regions of single-stranded viral RNA.

DCL4 appears to be the main producer of antiviral siRNAs against plus-strand RNA viruses, while DCL2 has a subordinate antiviral role when DCL4 activity is inhibited (Blevins *et al.*, 2006; Bouché *et al.*, 2006; Deleris *et al.*, 2006; Du *et al.*, 2007; Fusaro *et al.*, 2006; Garcia-Ruiz *et al.*, 2010; Qu *et al.*, 2008). However, DCL2 appears to be also involved in the generation of siRNAs from specific regions of viral RNAs (Donaire *et al.*, 2008), and also in silencing in systemic tissues (Garcia-Ruiz *et al.*, 2010). Although there are some indications that DCL3 contributes to antiviral defense, especially when DCL4 is inactivated (Blevins *et al.*, 2006; Diaz-Pendon *et al.*, 2007; Donaire *et al.*, 2008; Garcia-Ruiz *et al.*, 2010; Qu *et al.*, 2008), this contribution does not appear to be very relevant (Deleris *et al.*, 2006; Garcia-Ruiz *et al.*, 2010). Surprisingly, some data suggest that DCL1 might repress antiviral RNA silencing by negatively regulating DCL3 and DCL4 expression (Qu *et al.*, 2008). All four *Arabidopsis* DCLs mediate the defense response against DNA viruses (Blevins *et al.*, 2006; Moissiard & Voinnet, 2006). While DCL2, DCL3, and DCL4 are directly responsible for siRNA synthesis, DCL1 facilitates viral siRNA production by other DCLs in the CaMV infection, likely by excising the highly-structured 35S leader and thereby enhancing its accessibility to further DCL cleavage (Moissiard & Voinnet, 2006). However, the detection of 21-nt siRNAs in infected *dcl2/dcl3/dcl4* mutant plants suggests that DCL1 may also directly mediate siRNA synthesis in geminiviral and caulimoviral infections (Blevins *et al.*, 2006; Moissiard & Voinnet, 2006).

Only a fraction of the siRNAs produced in an RNA silencing reaction result from DCL-mediated cleavage of the initial trigger. These primary siRNAs participate in an RDR-mediated amplification phase that gives rise to secondary siRNAs (Moissiard *et al.*, 2007). For instance, recent results show that both RDR1 and RDR6 *Arabidopsis* enzymes contribute hugely to the biogenesis of siRNAs derived from CMV (Wang *et al.*, 2010), whereas the RDR1 action seems to be more relevant during TuMV infection (Garcia-Ruiz *et al.*, 2010). Furthermore, the bulk of antiviral siRNAs could be secondary siRNAs derived from either the processing of viral dsRNAs generated by endogenous RDRs or from direct RDR products (Diaz-Pendon *et al.*, 2007; Donaire *et al.*, 2008; Qu *et al.*, 2008; Wang *et al.*, 2010). It has

been also recently demonstrated that endogenous 22-nt miRNAs trigger RDR6-dependent secondary siRNAs (Chen *et al.*, 2010; Cuperus *et al.*, 2010), raising the possibility that virus-derived siRNAs with 22-nt of length, produced by the action of DCL2, could have an important role in the amplification step of antiviral silencing.

Dicer-mediated viral dsRNA processing could, in principle, be sufficient to disturb viral replication. However, although viral siRNAs accumulated to similar levels in *dcl2/dcl4*, *dcl2/dcl3* and *dcl3/dcl4 Arabidopsis* mutants infected with TRV, the *dcl2/dcl4* mutant, which is deficient in RISC-mediated trans silencing activity, displayed enhanced symptoms and viral titers compared to the trans silencing–active *dcl2/dcl3* and *dcl3/dcl4* mutants, suggesting that RISC activity contributes significantly to RNA silencing-mediated antiviral defense (Deleris *et al.*, 2006). In line with this suggestion, it has been demonstrated that viral siRNA-programmed RISC cleaves viral RNA, preferentially in discrete hot spots (Pantaleo *et al.*, 2007). Moreover, because plant miRNAs and siRNAs partially act via translational inhibition (Brodersen *et al.*, 2008), it is conceivable that the translational inhibition of viral mRNAs by viral siRNA-loaded RISC could contribute to siRNA-mediated antiviral defense. However, experimental evidence supporting this assumption is lacking (Ding & Voinnet, 2007).

The enhanced susceptibility of *ago1* mutants to viral infections (Morel *et al.*, 2002) and the co-immunoprecipation of AGO1 and viral siRNAs (Zhang *et al.*, 2006) suggest that AGO1 could be the main antiviral slicer in plants. However, at least AGO2 and AGO5 also recruit viral siRNAs (Takeda *et al.*, 2008), and AGO7 can contribute to the efficient clearance of viral RNAs (Qu *et al.*, 2008). These results suggest that different viral siRNA–loaded RISC complexes may perform more or less specialized functions in viral infections.

DCL3-derived 24-nt siRNAs guide DNA/histone methylation and transcriptional repression (Xie *et al.*, 2004). Thus, the impact of 24-nt siRNAs on DNA virus accumulation, in contrast with their minimal relevance in RNA virus infections, could result from the nuclear targeting of viral promoters, which causes transcriptional inactivation of the viral genomic DNA (Moissiard & Voinnet, 2006). Consistent with this hypothesis, transcriptional silencing associated with promoter methylation has been found in geminivirus promoter–driven transgenes following homologous virus infection (Seemanpillai *et al.*, 2003). In addition, bombardment or transgenic expression of dsRNAs homologous to the viral promoter sequences can enhance resistance to geminivirus infections (Pooggin & Hohn, 2003; Vanderschuren *et al.*, 2007). A more direct evidence that transcriptional silencing may be used as an antiviral defense in plants is the demonstration that geminiviral genome is methylated in infected plants (Raja *et al.*, 2008; Rodríguez-Negrete *et al.*, 2009), and that

Arabidopsis mutants defective on key methylation factors are hypersensitive to geminivirus infection (Raja *et al.*, 2008).

RISC loaded with virus-derived siRNAs can target not only viral sequences but also host genes with local sequence similarity to the viral genome. The resulting interference with host homeostasis could contribute to symptom induction. Consistent with this suggestion, transgenic plants expressing hairpin RNA derived from *Potato spindle tuber viroid* developed symptoms similar to those of plants infected with the viroid itself (Wang *et al.*, 2004). Moreover, bioinformatics analyses predict that a large number of host transcripts might be targeted by siRNAs derived from the CaMV 35S leader sequence, and the down-regulation of some of these transcripts has been experimentally verified in CaMV-infected plants (Moissiard & Voinnet, 2006). Although the host gene silencing by some viral siRNA can be irrelevant for the course of viral infection, other siRNAs may have been evolutionarily selected to disturb host defense factors or to promote plant physiological changes that favour viral proliferation (Ding & Voinnet, 2007). Although there is abundant evidence that RNA silencing mediated by viral-encoded miRNAs is relevant in mammalian viral infections (Gottwein & Cullen, 2008; Sullivan & Ganem, 2005b), it remains unclear whether virus-encoded siRNAs play similar roles in plant viral infections. Interestingly, recent data suggest that virus-derived siRNAs can be used by plant viruses to control their own parasites, namely satellite RNAs (Pantaleo & Burgyán, 2008).

The possible role of cellular small RNAs in antiviral defense remains controversial. There is no experimental evidence for the direct effects of endogenous small RNAs in natural plant viral infections. Artificial miRNAs have been shown to confer viral resistance in transgenic plants (Duan *et al.*, 2008; Niu *et al.*, 2006; Qu *et al.*, 2007) and endogenous miRNAs can target engineered plant viruses (Simón-Mateo & García, 2006). However, these recombinant viruses readily escape RNA silencing interference through mutations within the miRNA target sequence. Cellular miRNAs targeting animal viruses have been characterized (Huang *et al.*, 2007; Lecellier *et al.*, 2005; Otsuka *et al.*, 2007; Pedersen *et al.*, 2007; Triboulet *et al.*, 2007). However, rather than acting as a host antiviral strategy, RNA silencing mediated by these miRNAs may be used by the virus to avoid excessive proliferation that could kill the cell and potentially harm long-term viral survival (García & Simón-Mateo, 2006). This hypothesis would explain why the target sequences of these miRNAs have been evolutionarily conserved in the viral genomes. An example that clearly illustrates that cellular RNA silencing pathways can facilitate viral infection is the requirement of the liver-specific human miR-122 for hepatitis C viral replication (Jopling *et al.*, 2005).

Since RNA silencing is activated upon viral replication, the cell-autonomous branch of RNA silencing should be unable to prevent the infection, and only able to limit accumulation, in primary inoculated cells. However, RNA silencing also has non–cell autonomous arms, which, if activated faster than the spread of infectious viral RNA, can prime distant tissue to resist viral invasion. The cell-to-cell spread of RNA silencing can interfere with short-range viral movement between cells (Bayne *et al.*, 2005). In addition, systemic silencing, which depends on RDR6-mediated amplification, appears to have a major antiviral impact, especially by preventing meristem invasion by the virus (Schwach *et al.*, 2005). Viral exclusion from meristematic stem cells is especially important for antiviral defense, as it can facilitate plant recovery and help to prevent seed transmission. Two not mutually exclusive mechanisms have been proposed for the RNA silencing–mediated meristem exclusion of plant viruses. In one mechanism, a virus-specific systemic silencing signal reaches the meristem and primes a silencing response, preventing virus accession (Schwach *et al.*, 2005). In the second mechanism, silencing priming involves the transient presence of the virus in the meristematic cells (Martín-Hernández & Baulcombe, 2008).

I.1.4 Plant viruses fight back. Molecular mechanisms of RNA silencing suppression

The simple observation that viruses can infect plants suggests that they have found specific strategies to escape defense barriers such as RNA silencing–mediated resistance. Perhaps the most ancient and efficient way to protect the viral genetic material from plant attack is virion assembly; for instance, virions of TMV can persist for many years both inside and outside of cells (Dorokhov, 2007). Similarly, the compartmentalization of viral genetic material is a useful strategy for avoiding viral recognition by the defensive machinery; hence, most plant viruses employ membrane vesicles to replicate (Buck, 1996). The production of highly-structured RNAs appears to be a suitable strategy to avoid degradation. For instance, viroids have some structural protection against RISC (Carbonell *et al.*, 2008; Itaya *et al.*, 2007), and one umbraviral protein can form filamentous ribonucleoprotein complexes that protect the genomic RNA (Taliansky *et al.*, 2003).

In 1998, three different groups independently discovered that potyviral HCPro can suppress RNA silencing (Anandalakshmi *et al.*, 1998; Brigneti *et al.*, 1998; Kasschau & Carrington, 1998). Since then, different approaches have been used to identify a large number of viral RSSs, indicating that the expression of proteins with RNA silencing suppression activity is a common strategy used by plant viruses to disable this defense system in plants.

An updated, but not likely exhaustive, list of RSSs known as of August 2010 is presented in Table I1.

Currently known RSSs share neither conserved amino acid sequences nor evident structural similarities. Such exceptionally high divergences suggest that these viral factors are derived from independent and recent evolutionary events. In general, viral proteins with very different primary roles in infection have been co-opted as RSSs. Thus, silencing suppression activity has been observed in capsid proteins (CPs), movement proteins (MPs), vector transmission factors, RNA replication factors, transcriptional activators, and others. In addition, some viral RSS genes are overprinted on more ancient genes or result from gene duplication events, suggesting that they have been incorporated in the viral genome by typical gene expansion strategies (Li & Ding, 2006).

Table I1. Plant virus silencing suppressors

Virus genus	Virus name	RSSs	Proposed mechanism	Selected references
Positive-strand RNA viruses				
Bromoviridae				
Cucumovirus	*Cucumber mosaic virus*	2b	siRNA sequestering Ago1 inactivation	(Brigneti *et al*., 1998; Goto *et al*., 2007; Zhang *et al*., 2006)
	Tomato aspermy virus	2b	siRNA sequestering	(Chen *et al*., 2008a; Li *et al*., 1999)
Closteroviridae				
Closterovirus	*Beet yellows virus*	P21	siRNA sequestering	(Chapman *et al*., 2004; Reed *et al*., 2003; Ye and Patel, 2005)
	Beet yellow stunt virus	P22		(Reed *et al*., 2003)
	Citrus tristeza virus	P20		(Lu *et al*., 2004)
		P23		
		CP		
	Grapevine leafroll-associated virus-2	P24		(Chiba *et al*., 2006)
Criniviris	*Sweet potato chlorotic stunt virus*	P22*		(Kreuze *et al*., 2005)
		RNAse3	siRNA degradation	(Cuellar *et al*., 2010)
	Tomato chlorosis virus	P22		(Cañizares *et al*., 2008)
		CP		
		CPm		
Comoviridae				
Comovirus	*Cowpea mosaic virus*	S-CP		(Liu *et al*., 2004b)
Flexiviridae				
Potexvirus	*Potato virus X*	P25	Ago1 degradation	(Voinnet *et al*., 2000; Chiu *et al*., 2010)
Trichovirus	*Apple chlorotic leaf spot virus*	P50		(Yaegashi *et al*., 2007a; Yaegashi *et al*., 2008)
Vitivirus	*Grapevine virus A*	P10	siRNA sequestering	(Chiba *et al*., 2006; Zhou *et al*., 2006)
Luteoviridae				
Polerovirus	*Beet western yellows virus*	P0	Ago1 degradation	(Baumberger *et al*., 2007;

				Bortolamiol *et al*., 2007; Pazhouhandeh *et al*., 2006; Pfeffer *et al*., 2002)
	Cucurbit aphid-born yellows virus	P0		(Pfeffer *et al*., 2002)
	Potato leafroll virus	P0		(Pfeffer *et al*., 2002)
Potyviridae				
Ipomovirus	*Cucumber vein yellowing virus*	P1b	siRNA sequestering	(Valli *et al*., 2008)
	Sweet potato mild mottle virus	P1	Ago1 interaction	(Giner *et al*., 2010)
	Cassava brown streak virus	P1		(Mbanzibwa *et al*., 2009)
Potyvirus	*Tobacco etch virus*	HCPro	siRNA sequestering rgs-CAM interaction	(Anandalakshmi *et al*., 2000; Anandalakshmi *et al*., 1998; Kasschau and Carrington, 1998; Lakatos *et al*., 2006; Mérai *et al*., 2006)
	Potato virus Y	HCPro		(Brigneti *et al*., 1998)
	Turnip mosaic virus	HCPro		(Kasschau *et al*., 2003)
	Plum pox virus	HCPro		(Tenllado *et al*., 2003)
	Zucchini yellow mosaic virus	HCPro	siRNA sequestering	(Lin *et al*., 2007; Shiboleth *et al*., 2007)
	Sugarcane mosaic virus	HCPro		(Zhang *et al*., 2008a)
Tritimovirus	*Wheat streak mosaic virus*	P1		(Stenger *et al*., 2007)
Tombusviridae				
Aureusvirus	*Pothos latent virus*	P14	siRNA sequestering dsRNA protection	(Mérai *et al*., 2005)
Carmovirus	*Turnip crinkle virus*	CP (P38)	Ago1 interaction siRNA sequestering dsRNA protection	(Azevedo *et al*., 2010; Mérai *et al*., 2006)
	Hibiscus chlorotic ringspot virus	CP		(Meng *et al*., 2006)
	Pelargonium flower break virus	CP	siRNA sequestering	(Martínez-Turiño & Hernández, 2009)
Tombusvirus	*Cymbidium ringspot virus*	P19	siRNA sequestering	(Lakatos *et al*., 2004; Silhavy *et al*., 2002)
	Tomato bushy stunt virus	P19	siRNA sequestering	(Omarov *et al*., 2006; Voinnet *et al*., 1999)
Dianthovirus	*Red clover necrotic mosaic virus*	Replication complex		(Takeda *et al*., 2005)
		MP		(Powers *et al*., 2008a; Powers *et al*., 2008b)
Tymoviridae				
Tymovirus	*Turnip yellow mosaic virus*	P69		(Chen *et al*., 2004)
Unassigned				
Benyivirus	*Beet necrotic yellow vein virus*	P14		(Dunoyer *et al*., 2002)
		P31		(Rahim *et al*., 2007)
Cheravirus	*Apple latent spherical virus*	CP Vp20		(Lu *et al*., 2004; Yaegashi *et al*., 2007a; Yaegashi *et al*., 2007b)
Furovirus	*Soil-borne wheat mosaic virus*	19K		(Te *et al*., 2005)
Hordeivirus	*Barley stripe mosaic virus*	γb		(Yelina *et al*., 2002)
	Poa semilatent virus			
Pecluvirus	*Peanup clump virus*	P15	siRNA sequestering	(Dunoyer *et al*., 2002; Mérai *et al*., 2006)
Sobemovirus	*Rice yellow mottle virus*	P1		(Voinnet *et al*., 1999)
	Cocksfoot mottle virus	P1		(Sarmiento *et al*., 2007)
Tobamovirus	*Tobacco mosaic virus*	126K	siRNA sequestering	(Csorba *et al*., 2007; Ding *et al*., 2004; Mérai *et al*., 2006)
	Tomato mosaic virus	130K		(Kubota *et al*., 2003)
	Pepper mild mottle virus	130K		(Tsuda *et al*., 2007)

Tobravirus	*Tobacco rattle virus*	16K		(Liu *et al*., 2002; Martín-Hernández and Baulcombe, 2008; Martínez-Priego *et al*., 2008)
Negative-strand RNA viruses				
Bunyaviridae				
Tospovirus	*Tomato spotted wilt virus*	NSs		(Bucher *et al*., 2003; Takeda *et al*., 2002)
Unassigned				
Tenuivirus	*Rice hoja blanca virus*	NS3	siRNA sequestering	(Bucher *et al*., 2003; Hemmes *et al*., 2007; Schnettler *et al*., 2008)
Double-strand RNA viruses				
Reoviridae				
Phytoreovirus	*Rice dwarf virus*	Pns10		(Cao *et al*., 2005)
DNA viruses				
Caulimoviridae				
Caulimovirus	*Cauliflower mosaic virus*	P6	DRB4 inhibition	(Haas *et al*., 2008; Love *et al*., 2007, Shivaprasad *et al*., 2008)
Geminiviridae				
Begomovirus	*African cassava mosaic virus*-CM	AC4	Sequestering of mature small RNAs	(Chellappan *et al*., 2005)
	African cassava mosaic virus-KE	AC2		(Voinnet *et al*., 1999)
	East African cassava mosaic virus Cameroon	AC2		(Vanitharani *et al*., 2004)
	Indian cassava mosaic virus	AC2		(Vanitharani *et al*., 2004)
	Mungbean yellow mosaic virus	AC2	Transactivation of host factors	(Trinks *et al*., 2005; Vanitharani *et al*., 2004)
	Sri Lankan cassava mosaic virus	AC2		(Vanitharani *et al*., 2004)
	Tomato golden mosaic virus	AC2	ADK inhibition	(Wang *et al*., 2005; Wang *et al*., 2003)
	Tomato leaf curl Java virus DNAß02	AC2		(Kon *et al*., 2007)
		ßC1		(Kon *et al*., 2007)
	Tomato yellow leaf curl China virus	AC2		(van Wezel *et al*., 2002)
	Tomato yellow leaf curl China virus Y10ß	ßC1		(Cui *et al*., 2005)
	Tomato yellow leaf curl virus-Is	V2	SGS3 inhibition	(Glick *et al*., 2008)
Curtovirus	*Beet curly top virus*	C2	ADK inhibition	(Wang *et al*., 2005; Wang *et al*., 2003)

* P22 is absent from some SPCSV.

Some RSSs, such as tombusviral P19, potyviral HCPro, or carmoviral P38 (Ding & Voinnet, 2007), target the core steps of RNA silencing by affecting Dicer or RISC activities. Others target more peripheral branches of the process (Fig. I1). For instance, tymoviral P69 and tospoviral NSs reportedly interfere with the dsRNA synthesis required for the generation of secondary viral siRNAs (Chen *et al.*, 2004a; Ding & Voinnet, 2007; Takeda *et al.*, 2002). The CP of the closterovirus CTV, one of the CPs, Vp20, of the cheravirus ALSV, and the MP P50 of the trichovirus ACSLV, which do not affect intracellular silencing, suppress intercellular silencing spread (Lu *et al.*, 2004; Yaegashi *et al.*, 2007a; Yaegashi *et al.*, 2008).

It is not unusual for RSSs to target multiple steps of the silencing process, thereby enhancing the suppression effectiveness. For instance, cucumoviral 2b inhibits the activity of AGO1-containing RISC (Zhang *et al.*, 2006), but also interferes with the propagation of the systemic RNA silencing signal (Guo & Ding, 2002). Another strategy used by plant viruses to improve the suppression of RNA silencing-mediated host defensive responses is the production of several RSSs. These RSSs likely have additive or synergistic effects by targeting different silencing steps. Such a strategy might be used by CTV (Lu *et al.*, 2004) and the crinivirus ToCV (Cañizares *et al.*, 2008), which have at least three distinct RSSs, and the dianthovirus RCNMV (Powers *et al.*, 2008a) and the benyvirus BNYVV (Rahim *et al.*, 2007), for which two different silencing suppression activities have been reported. Potyviral P1 and a crinivirus-encoded RNase III-like protein were reported to enhance the activities of the RSSs HCPro (Pruss *et al.*, 1997; Rajamäki *et al.*, 2005) and p22 (Kreuze *et al.*, 2005), respectively, whereas they lacked detectable RNA silencing suppression activity by themselves. However, more recent results have revealed an RNA silencing suppression activity of SPCSV RNAse3 (Cuellar *et al.*, 2009), and some results from our laboratory suggest that potyviral P1 could have also an independent silencing suppression activity, perhaps restricted to the natural virus infection.

Details of the molecular mechanisms underlying silencing suppression activity are known for only a few RSSs, but point to RSSs having very specific mechanisms of action. However, some common biochemical properties appear to have been adopted by completely unrelated RSSs by convergent evolution. We will next summarize the data available on different silencing suppression strategies used by known RSSs *Interaction with RNA*

Given the key role played by RNAs in the antiviral silencing pathway, it is conceivable that any mechanism that promotes the non-specific degradation of RNA components of the RNA silencing pathway or prevents functional interactions involving these factors will inhibit the silencing cascade (Fig. I2A-D). Indeed, many RSSs display RNA-binding activities (Mérai *et al.*, 2006), and X-ray crystallography indicates that different RSSs have adopted very diverse folds to bind RNA (Chao *et al.*, 2005; Chen *et al.*, 2008; Lingel *et al.*, 2005; Vargason *et al.*, 2003; Ye *et al.*, 2003; Ye & Patel, 2005).

The cleavage of dsRNAs by Dicer-like enzymes is a core step of RNA silencing. In principle, the interaction of a protein with a long dsRNA precursor could protect it from Dicer digestion (Fig. I2A). This silencing suppression mechanism has been demonstrated for RSSs of insect nodaviruses (Lu *et al.*, 2005; Sullivan & Ganem, 2005a) and cripaviruses (van Rij *et al.*, 2006). The plant RSSs P14 of the aureusvirus PoLV and P38 of the carmovirus TCV,

which interact with dsRNAs without length preference (Mérai *et al.*, 2006), are also thought to use this mechanism of silencing suppression. Since these proteins can bind to RNA duplexes of a small size, they may also mediate silencing suppression by sequestering viral siRNAs (Chao *et al.*, 2005; Mérai *et al.*, 2006). Specific binding to small RNAs (Fig. I2B) appears to be a more common strategy of plant virus RSSs (Chapman *et al.*, 2004; Csorba *et al.*, 2007; Goto *et al.*, 2007; Kurihara *et al.*, 2007; Martínez-Turiño & Hernández, 2009; Mérai *et al.*, 2006), and experimental evidence supports the notion that siRNA binding by tombusviral P19, closteroviral P21, potyviral HCPro, tenuiviral NS3, and tobamoviral P122 may interfere with the loading of ds-siRNAs in RISC complexes (Csorba *et al.*, 2007; Hemmes *et al.*, 2007; Lakatos *et al.*, 2006).

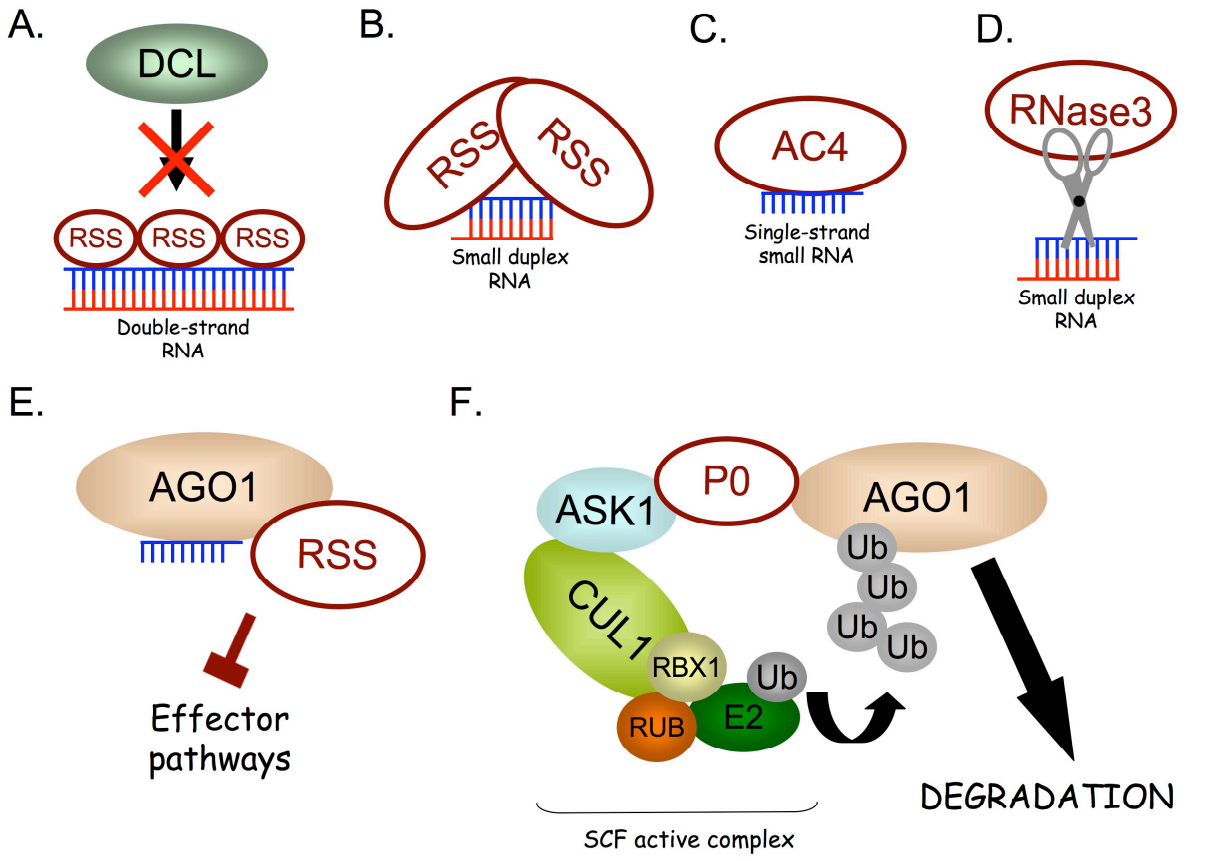


Figure I2. Schematic representation of diverse antisilencing mechanisms used by viral RSSs. (A) RSSs able to bind long RNA duplexes can protect these molecules against DCL processing. (B) Sequestering of ds small RNA by RSS complexes interferes with effector activities. (C) Begomoviral AC4 is the unique RSS described to date showing high affinity by ss small RNAs. (D) SPCSV RNase3 cleaves ds small RNAs to produce non-functional shorter small RNAs. (E) Direct interaction between RSSs and AGO1 prevents the action the RISC effector complex. (F) Poleroviral P0 is a F-Box protein able to form a SCF active complex that mediates AGO1 degradation.

The structural basis of the siRNA-binding activity of tombusviral P19 has been precisely defined. P19 forms a homodimer that acts as a molecular caliper to specifically select 21- to 22-nt siRNAs (Vargason *et al.*, 2003; Ye *et al.*, 2003). These results suggest that P19 has adapted its binding features to recognize, by size selectivity, viral siRNAs produced in the course of tombusviral infection, but not longer siRNA duplexes. Such size specificity could prevent side effects of P19 RNA binding on diverse endogenous processes, for instance chromatin modifications by DNA methylation, where longer siRNAs play a crucial role. However, given the structural similarity between endogenous miRNAs and viral siRNAs, miRNA species are also sequestered by P19, promoting inhibition of the miRNA silencing pathway and consequent alterations of the developmental patterns in infected plants (see below).

Although the siRNA binding by different RSSs is likely functionally equivalent, there appear to be some specific peculiarities. For instance, while 2-nt 3' overhangs typical of Dicer-derived small RNAs are not essential for siRNA binding to tombusviral P19 (Lakatos *et al.*, 2006) and tenuiviral NS3 (Hemmes *et al.*, 2007), they do appear to significantly contribute to the siRNA binding of closteroviral P21, potyviral HCPro (Lakatos *et al.*, 2006), and tobamoviral P122 (Csorba *et al.*, 2007). Moreover, crystallographic data show that when binding siRNAs, cucumoviral 2b adopts a structure that is completely different from that of tombusviral P19, thereby justifying the different RNA length preferences of these proteins (Chen *et al.*, 2008).

Some RSSs that bind small RNAs, namely HCPro, P38, P19, P122, and 2b, interfere with RNA silencing amplification, thus preventing secondary siRNA accumulation (Csorba *et al.*, 2007; Mlotshwa *et al.*, 2008a; Moissiard *et al.*, 2007; Zhang *et al.*, 2008). Since the accumulation of primary siRNA is not affected, these siRNAs are thought to be inactivated by the aforementioned RSSs, either by physical sequestration or by another, yet unknown mechanism. Inactivation of the primary siRNAs might contribute to the blockade of secondary siRNA production, but this is probably not the only cause, since HCPro and P38 suppress secondary siRNA accumulation in the absence of known primary siRNAs during sense transgene silencing (Mlotshwa *et al.*, 2008a; Mlotshwa *et al.*, 2008b). HCPro and 2b down-regulate the expression of the RNA polymerase RDR6, a critical factor of the RNA silencing amplification process (Zhang *et al.*, 2008). This down-regulation may contribute to the suppression of secondary siRNA accumulation.

Plant small RNAs are protected from oligourydilation and degradation by methylation at their 3' termini (Yu *et al.*, 2005). Some RSSs that bind small RNAs can interfere with

small RNA methylation, which might contribute to inactivation of primary siRNAs and silencing suppression (Csorba *et al.*, 2007; Ebhardt *et al.*, 2005; Lózsa *et al.*, 2008; Vogler *et al.*, 2007; Yu *et al.*, 2006).

An exception to the typical binding of RSSs to dsRNAs is the protein AC4 of the begomovirus ACMV (Fig. I2C). AC4 interacts with the single-stranded forms of miRNAs and siRNAs, and likely acts downstream of RISC assembly (Chellappan *et al.*, 2005). This strategy of silencing suppression appears to be very specific to ACMV, since the AC4 protein of a closely related virus, EACMCV, neither binds single-stranded small RNAs nor displays silencing suppression activity. The RSS of EACMCV (AC2), and ACMV AC2, which has mild silencing suppression activity, also are unable to interact with the single-stranded forms of miRNAs and siRNAs (Chellappan *et al.*, 2005).

Cellular enzymes that interfere with RNA silencing by degrading siRNA duplexes are evolutionarily conserved in a wide range of eukaryotic organisms (Kennedy *et al.*, 2004). In *Arabidopsis*, the exoribonuclease SDN1 degrades single-stranded miRNAs (Ramachandran & Chen, 2008), but it is not known whether it affects viral siRNAs. Interestingly, this particular strategy of interfering with silencing machinery is displayed by the RNase3 protein from SPCSV (Fig. I2D), which use its endonuclease activity for cleaving ds-siRNAs of 21-, 22- and 24-nt of length to produce shorter small RNAs (~14-nt) with inefficient silencing activity (Cuellar *et al.*, 2009).

Interaction with host proteins

Many interactions between viral and host factors define the course of a viral infection. These interactions affect all steps of the viral life-cycle, not excepting suppression of RNA silencing. Core elements of RNA silencing pathways, such as Dicer-like or AGO proteins, appear to be excellent target candidates for RSS proteins. However, silencing suppression strategies based on interactions with host factors that participate directly or indirectly in other steps of RNA silencing are also suitable.

Interfering with slicing:

AGO1 appears to be a preferred target of RSSs (Fig. I2E and F). Interestingly, even though these RSSs target the same level of the silencing cascade (Fig. 1), they operate in quite different ways. Cucumoviral 2b directly binds to one surface of the PAZ-containing module of AGO1 *in vitro* and specifically disturbs AGO1 slicing activity in RISC reconstitution assays (Zhang *et al.*, 2006). Co-immunoprecipitation and -localization in the nucleus and

cytoplasmic foci have been used to confirm the specific CMV 2b-AGO1 interaction *in vivo* (Zhang *et al.*, 2006). However, recent results suggest that the siRNA binding capacity could be more relevant than AGO1 interaction for RNA silencing suppression activity of CMV 2b (Gonzalez *et al.*, 2010).

The strategy used by the P1 protein of the ipomovirus SPMMV and TCV P38 relies on mimicry of host-encoded glycine/tryptophane (GW)-containing factors. The GW motifs are conserved in many cellular proteins from different kingdoms and work as "AGO hooks" mediating direct interactions with Argonaute proteins (El-Shami *et al.*, 2007; Karlowski *et al.*, 2010; Lian *et al.*, 2009; Till *et al.*, 2007). Hence, SPMMV P1 and TCV P38 use their GW-motifs as hooks to bind and inhibit host AGO1 (Azevedo *et al.*, 2010; Giner *et al.*, 2010). Whereas different possibilities could explain the effects of these proteins on antiviral silencing, one of the most appealing hypotheses considers that these viral factors outcompete an essential yet-unidentified host protein required for correct RISC assembly/function (Azevedo *et al.*, 2010; Giner *et al.*, 2010). Interestingly, the study of TCV P38 also uncovered an endogenous AGO1-dependent regulatory network that controls the levels of DCL proteins. Thus, the previously described negative effect of TCV P38 on DCL4 activity would be a secondary effect promoted by the action of this suppressor on AGO1 activity (Azevedo *et al.*, 2010; Deleris *et al.*, 2006; Qu *et al.*, 2008).

In turn, poleroviral P0 contains an F-box–like domain and interacts with both SCF-like E3 ubiquitin ligase complex components (Pazhouhandeh *et al.*, 2006) and AGO1 (Baumberger *et al.*, 2007; Bortolamiol *et al.*, 2007). This observation suggests that the polerovirus RSS facilitates the polyubiquitination and subsequent degradation of AGO1 (Fig. I2F). However, in contrast with the typical action of SCF-type complexes, which direct target protein ubiquitination and subsequent degradation via the proteasome, P0-induced AGO1 decay appears to be proteasome-independent (Baumberger *et al.*, 2007; Csorba *et al.*, 2010). It has been raised the possibility that correct activity of AGO1 requires a specific pattern of ubiquitination, which would be disturbed by P0 either by dominant-negative inhibition of the host F box protein involved in the process or by directing a ineffectual pattern of ubiqitination (Baumberger *et al.*, 2007); however, a degradation AGO1 induced by destabilization of RISC complex prior to its formation cannot be rule out (Csorba *et al.*, 2010). In addition, poleroviral P0 mediates destabilization not only of AGO1 but also of other AGO proteins in *Arabidopsis*. This ability probably confers a broad range of activity on this RSS (Baumberger *et al.*, 2007). Similarly, direct interaction and negative effect on accumulation of AGO proteins have recently been reported for PVX P25 (Chiu *et al.*, 2010).

Interfering with dicing:

RSSs seemingly target dicing activity not only by protecting dsRNA substrates through dsRNA-binding proteins (see above), but also by directly inhibiting the enzymatic machinery involved in dsRNA processing. The replication machinery of the dianthovirus RCNMV is thought to deprive the RNA silencing machinery of its Dicer-like enzymes involved in siRNA biogenesis (Takeda *et al.*, 2005), but the molecular details of this silencing suppression strategy remain unknown. Another example where a dicing pathway is specifically inhibited by an RSS is the interaction of the CaMV protein P6 with DRB4, a dsRNA-binding protein that mediates the activity of the major plant antiviral factor DCL4 (Haas *et al.*, 2008; Shivaprasad *et al.*, 2008).

Interfering with the RNA silencing amplification step:

As mentioned above, the inactivation of siRNAs or of AGO1 appears to have a subsequent inhibitory effect on the RNA silencing amplification step. However, this step is probably the primary target of some RSSs, such as the P69 protein of the tymovirus TuYV (Chen *et al.*, 2004a) and the NSs protein of the tospovirus TSWV (Takeda *et al.*, 2002). These RSSs suppress the siRNA accumulation and silencing initiated by a sense RNA, but cannot prevent RNA silencing produced by an IR RNA that can be directly processed by DCL enzymes to yield large amounts of siRNAs. The specific targets of P69 and NSs in RNA silencing amplification remain unknown; however, the interaction of one protein involved in the amplification pathway, the coiled-coil domain protein SGS3 (Mourrain *et al.*, 2000), with the silencing suppressor V2 of the begomovirus TYLCV is relevant for RNA silencing suppression (Glick *et al.*, 2008). Surprisingly, TYLCV V2 does not suppress siRNA accumulation in a sense RNA silencing assay, which is expected to yield only secondary siRNAs (Zrachya *et al.*, 2007).

Cellular factors also appear to contribute to modulate the effects of RNA silencing by interfering with its RDR-mediated amplification phase. Host proteins that control RNA processing act as endogenous RSSs by preventing the formation of aberrant RNAs, which could be recognized by RDRs and introduced in the RNA silencing amplification pathway (Herr *et al.*, 2006). Similar endogenous RNA silencing suppression activity is manifested by cellular proteins that eliminate RNAs with anomalous features, such as RNA fragments produced by slicer activity that initiate the RNA silencing amplification process (Gazzani *et al.*, 2004; Gy *et al.*, 2007; Souret *et al.*, 2004).

Interactions with other host proteins:

RSSs also interact with host proteins that are not recognized components of the RNA silencing machinery. Using a yeast two-hybrid system, a tobacco rgsCaM was identified that interacts with HCPro and that by itself also has silencing suppression activity (Anandalakshmi *et al.*, 2000). Details on the molecular mechanism used by rgsCaM to suppress silencing, and on the relevance of HCPro/rgsCaM interactions for silencing suppression activities during viral infections, remain unknown. Recently, using similar approaches, RAV2 protein, an ethylene-inducible transcription factor, was identified as another HCPro interactor required not only for the silencing suppression activity of this viral factor, but also for that of TCV P38 (Endres *et al.*, 2010). The mechanism by which RSSs use RAV2 to suppress the RNA silencing is unknown.

AC2 (also named AL2) from the begomovirus TGMV and C2 (also named L2) from the curtovirus BCTV can suppress IR RNA–induced silencing (Wang *et al.*, 2005). The search for targets of these geminiviral factors has revealed that they interact with, and inactivate, a cytoplasmic adenosine kinase (ADK) (Wang *et al.*, 2003). Alternative methods of ADK inactivation, either through the addition of a chemical inhibitor or by co-delivering a construct expressing an IR ADK RNA, also suppress RNA silencing in a manner similar to these RSSs (Wang *et al.*, 2005). These results strongly suggest that ADK activity is required for RNA silencing, and that AC2 and C2 suppress silencing by a mechanism involving ADK inhibition. Since ADK plays a critical role in sustaining *S*-adenosylmethionine–dependent methyltransferase activity, ADK activity could be important for a defensive response mediated by viral gene transcriptional silencing by viral siRNA–induced promoter methylation (Wang *et al.*, 2005).

Trans-activation of host genes

An alternative way for viral proteins to suppress RNA silencing is to induce the expression or activation of endogenous negative regulators of RNA silencing. The primary function assigned to the begomovirus AC2 protein is the transcriptional activation of late viral genes (Sunter & Bisaro, 1992); however, as mentioned above, AC2 also has RNA silencing suppression activity (Vanitharani *et al.*, 2004; Voinnet *et al.*, 1999). Microarray experiments revealed that the accumulation of the transcript encoding WEL-1, a protein with homology to the 3'-5' exonuclease domain of the Werner syndrome protein, was strongly enhanced in *Arabidopsis* protoplasts expressing the AC2 protein from the begomoviruses MYMV and ACMV (Trinks *et al.*, 2005). This study also showed that WEL-1 displays intrinsic RNA

silencing suppression activity, for which two possible mechanisms have been suggested. First, WEL-1 might have a dominant-negative effect through interfering with an as yet uncharacterized silencing function of WEX, another Werner syndrome protein–related factor (Glazov *et al.*, 2003). Alternatively, as has been postulated for both endogenous and viral RSSs (see above), WEL-1 may mediate the degradation of RNA partners of the silencing pathway, such as siRNAs or slicer products recognized as substrates by RDRs during the silencing amplification process.

It remains unknown whether the two silencing suppression mechanisms described for begomoviral AC2 proteins, the inhibition of ADK and the transcriptional activation of a cellular RSS, are virus-specific or both could operate together. However, given that the transcription activation domain is not required for TGMV AC2 to suppress silencing (Wang *et al.*, 2005), but is essential for the anti-silencing activity of MYMV (Trinks *et al.*, 2005), AC2 proteins from different begomoviruses may adopt distinct strategies to suppress silencing. Since different experimental approaches have been followed for the different AC2 proteins, definitive conclusions await precise comparisons in common experimental systems.

I.2 The *Potyviridae* family of plant viruses*[2]

I.2.1 General considerations and classification

The family *Potyviridae* owes its name to the type member *Potato virus Y*. It is considered the largest group of plant viruses, being included within the picorna-like supergroup, together with viruses exhibiting a similar genome belonging to the families *Comoviridae*, *Sequiviridae*, *Picornaviridae*, and *Hypoviridae* (Berger *et al.*, 2005). Virtually every crop of agronomic or horticultural use can suffer losses caused by one or more members of this family, producing, in many occasions, the complete crop failure. As a consequence, and bearing in mind its sheer size, the family is often considered the most important group of plant viruses from an economic standpoint. Furthermore, their control involves significant costs. Many members of the *Potyviridae* are present worldwide, and new emerging diseases are also common.

Within the family, genera can be differentiated by biological criteria, mainly transmission by specific vectors complemented by molecular data. The *Potyviridae* includes the genus *Potyvirus* with more than one hundred members (having almost the same number of tentative members), characterized as being aphid transmitted. Other viruses in the family are

*[2] This section is based on **López-Moya, J. J., Valli, A. & García, J. A.** Ibid.Potyviridae, p. http://www.els.net/ [DOI: 10.1002/9780470015902.a9780470000755.pub9780470015902].

distributed in the genus *Macluravirus,* which are also transmitted by aphids, the genus *Ipomovirus* with whitefly transmitted members, and the genera *Rymovirus* and *Tritimovirus*, transmitted by mites. All these viruses have monopartite genomes, while another genus within the family, the genus *Bymovirus,* includes viruses with bipartite genomes that are transmitted by plasmodiophorids. As the number of available sequences builds up, it is likely that new taxons might be necessary to account for peculiarities in certain viruses; hence, the addition of two new genera, termed putatively as *Brambyvirus* and *Poacevirus* (or *Susmovirus*) is currently under evaluation. Different genera comprising this family, indication of their type members and some characteristics used in taxonomical discrimination, are listed in table I2.

Table I2. Recognized genera of the family *Potyviridae*.

Genus	Type member	Genome	Vector type
Bymovirus	*Barley yellow mosaic virus*	Bipartite	Plasmodiophorid (Polymyxa spp.)
Ipomovirus	*Sweet potato mild mottle virus*	Monopartite	Whiteflies
Macluravirus	*Maclura mosaic virus*	Monopartite	Aphids
Potyvirus	*Potato virus Y*	Monopartite	Aphids
Rymovirus	*Ryegrass mosaic virus*	Monopartite	Mites (Abacarus spp.)
Tritimovirus	*Wheat streak mosaic virus*	Monopartite	Mites (Aceria spp.)

I.2.2 Structure and properties of the viral genome

Viruses of the *Potyviridae* family have positive-sense ssRNA genomes, which are encapsidated into flexuous-rod particles of approximately 11-14 nm in diameter and 680-950 nm in length formed by repeated units of a single structural coat protein (CP) (Fig. I3A); in the case of bymoviruses, the two genomic RNA are in one of two particles of different length.

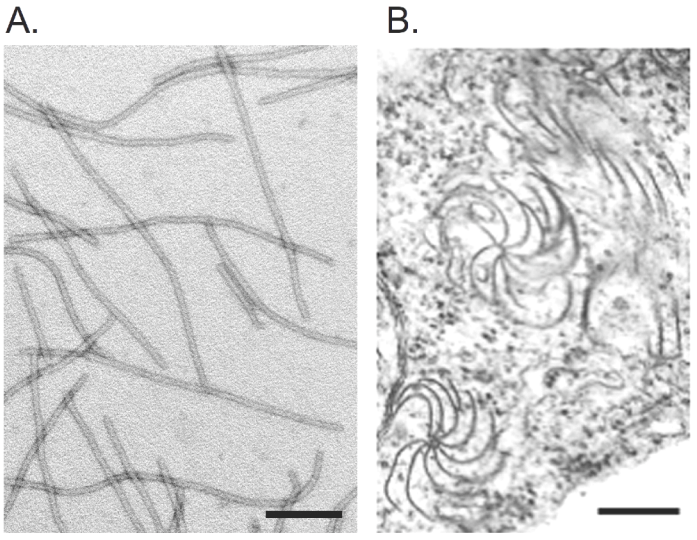


Figure I3. Distinctive structural features of potyviruses. (A) Electron microscopy of flexuous-rod particles of PPV. (B) Pinwheel inclusions in the cytoplasm of a *Nicotiana benthamiana* cell infected with PPV (courtesy of D. López-Abella, CIB, Madrid, Spain). Bar equals 200 nm.

The positive (+) sense genomic RNA of monopartite members of the family ranges from 8.2 kb to nearly 11 kb in size. The 5'-end of the genome is attached covalently to a viral protein of approximately 24 kDa (VPg), and the 3'-end is polyadenylated with a variable number of adenosines (An) (Fig. I4). The RNA contains a large ORF that encodes a polyprotein of about 350 kDa, comprising, in most of the cases, the following gene products from N- to C-termini: P1, HCPro, P3, 6K1, CI, 6K2, NIa(VPg+Pro), NIb and CP (Fig. I4A). Bymoviruses have a similar structure although the genome is divided between two components, each encoding a polyprotein, with two products (P2-1 and P2-2) in the shorter one, and seven in the longer one corresponding to the P3 onwards gene products of the polyprotein from monopartite members of the family (Fig. I4D). Other differences in the genome structure are found in some viruses still included in the ipomovirus genus, CVYV, SqVYV and CBSV, where the HCPro cistron is missing in the three cases, while two P1 serine-proteases, termed P1a and P1b, are present in CVYV and SqVYV (Fig. I4B and C). Exceptionally, CBSV genome contains an additional cistron located between the NIb and CP coding sequences, named HAM1 (Fig. I4C). The function of this viral gene, given its homology to cellular genes coding for Maf/HAM1 NTP pyrophosphatases, could be to intercept noncanonical NTPs to reduce mutation rates of viral RNA (Mbanzibwa *et al.*, 2009).

Despite the abundant sequence information available for many years, only recently the presence of a second short ORF, termed *pipo* by its discoverers, was predicted embedded within the P3 region and well conserved in a similar position in all members of the family (Fig. I3). This ORF could theorically yield a different product after a +2 frameshift, and indeed a fusion product with the upstream portion of P3 after frameshift was found in TuMV infected plants, and termed P3N+PIPO (Chung *et al.*, 2008).

A typical potyvirus genome starts with a 5' non-coding region (NCR), less than 200 nt, containing regulatory elements and acting as enhancer of translation through mechanisms still not fully understood (Carrington & Freed, 1990). Studies in PPV showed that translation takes place by cap-independent leaky scanning, with most of the region being dispensable for infectivity, although it contributes to viral competitiveness and pathogenesis. In the case of the 5'-NCR of TEV, the presence of an internal initiation site has been suggested (Zeenko & Gallie, 2005).

Another NCR of about 200 bp is located at the 3' end of the genome before the polyA tail. Putative RNA structures in this region participate in pathogenesis, and also the existence of elements necessary for infectivity has been proposed (Haldeman-Cahill *et al.*, 1998).

The polyprotein(s) encoded by a potyvirus genome is co- and post-translationally cleaved by the action of viral-encoded proteases to produce the mature protein products (Adams *et al.*, 2005a). The polyprotein of viruses from *Potyvirus* genus, for instances, is processes by three viral proteases. Thus, P1 and HCPro cleave at their C-termini autocatalytically, whereas the C-terminal protease domain of NIa catalyses the processing of the rest of the polyprotein (Fig. I3A). Intriguingly, *in vitro* analysis of P1 self-cleavage showed that, in different manner than the other proteases, it only takes place in wheat germ extract but not in reticulocyte lysate system, suggesting that a plant co-factor is require for the protease activity of P1 (García *et al.*, 1993; Verchot *et al.*, 1992). In case of CVYV, SqVYV and CBSV ipomoviruses the maturation process mediated by viral proteases must be different given that they lack of the HCPro cistron. In case of bymoviruses, only two proteases are required.

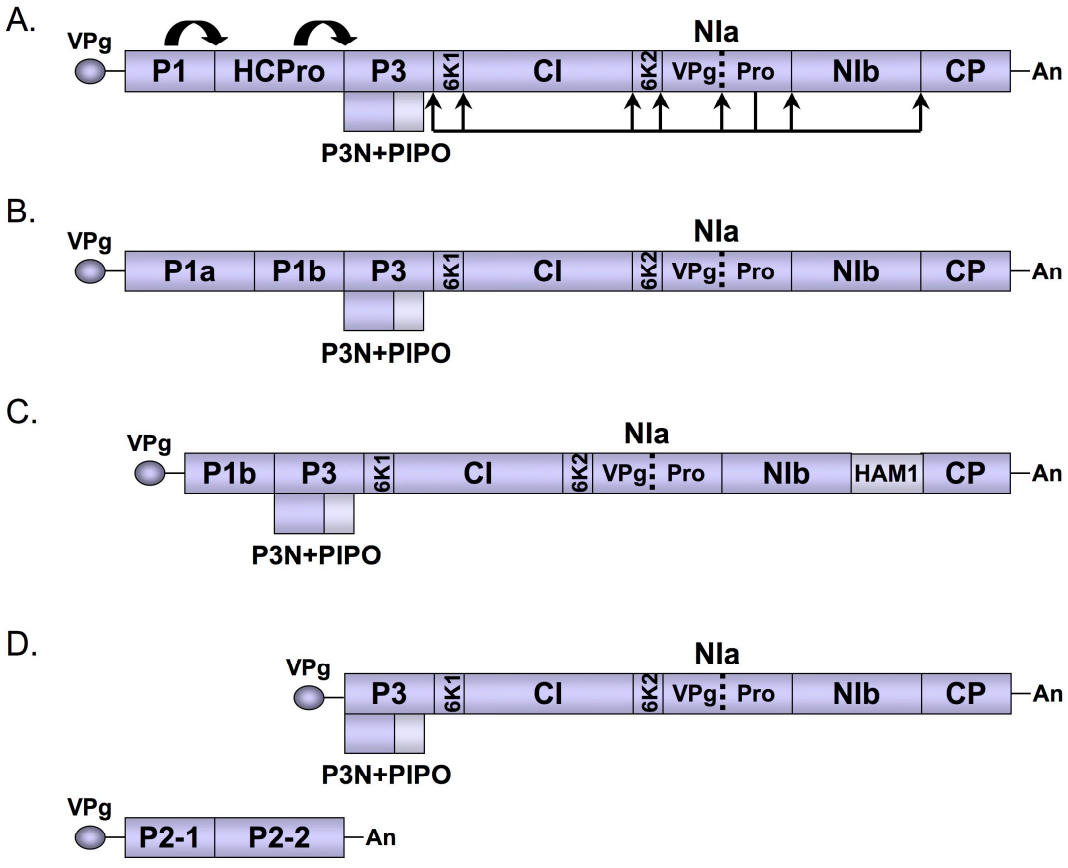


Figure I4. Genomic maps of viruses in the family *Potyviridae*. The genomic ssRNAs carry a covalently linked VPg protein at the 5' end, shown as circles, and a poly A tail (An) at the 3' end. ORFs are indicated as boxes, with their names, and divided by vertical lines. Arrows indicate cleavage sites and the protease responsible for each cleavage. (A) Organization of the gene products in potyviruses, tritimoviruses, rymoviruses and SPMMV ipomovirus. (B) A variant of the above organization with the absence of HCPro cistron and two P1 serine-proteases in tandem (P1a-P1b), as in CVYV and SqVYV ipomoviruses. (C) Another variant of the above organization with the absence of the HCPro region, as in the case of CBSV ipomovirus. Note that it also contains an additional cistron (HAM1) between NIb and CP. (D) Organization of genomics RNAs of a bipartite *Bymovirus*.

I.2.3 Functions of gene products

Replication of the genome of these viruses appears to be typical for (+) ssRNA viruses, where the plus strand is transcribed to produce a (–) RNA strand, which then serves as template for the production of progeny (+) strand RNA molecules. This process probably occurs the membraneous structures in the cytoplasm of infected cells (Cotton *et al.*, 2009; Wei *et al.*, 2010b). Most if not all of the mature viral proteins are multifunctional, and interactions among them are also common (Guo *et al.*, 2001).

- P1:

The P1 is an exclusive factor of monopartite members of *Potyviridae* family, not being present in bymoviruses. It is the most divergent protein with regard to both length and amino acid sequence (Adams *et al.*, 2005b); however, at the C-terminal region, they contain a relatively conserved serine protease domain that cleaves the viral polyprotein at the junction between the P1 C-terminus and the adjacent product (Fig. 3A) (Verchot *et al.*, 1991). This protein acts as an accessory factor for genome amplification (Verchot & Carrington, 1995). Although the function of P1 in infections is still unknown, there is some indication that P1s from potyviruses can strengthen the ability of HCPro to suppress the RNA silencing (Kasschau & Carrington, 1998; Rajamäki *et al.*, 2005) and enhance the pathogenicity of heterologous plant viruses in synergistic interactions (Pruss *et al.*, 1997).

Frequent recombinations can be found when P1 sequences are analyzed, a fact that prompted consideration the possible involvement of this product in host range specificity and adaptation. As mentioned, some ipomoviruses have two P1 copies, which appear to have not only different origins, but also different functions.

- HCPro:

HCPro is a multifunctional protein (Urcuqui-Inchima *et al.*, 2001), being involved in genome amplification, long distance movement within the plant and aphid transmission. It is a papain-like cysteine protease, which cleaves the polyprotein at the junction between the HCPro C-terminus and the adjacent product (Fig. 3A). Some structural studies with LMV and TEV have demonstrated the HCPro capacity to assemble in pairwise oligomeric forms (Plisson *et al.*, 2003; Ruiz-Ferrer *et al.*, 2005). Interactions with both viral and host factors have also been described (Anandalakshmi *et al.*, 2000; Blanc *et al.*, 1997; Cheng *et al.*, 2008; Endres *et al.*, 2010; Guo *et al.*, 2003; Jin *et al.*, 2007a; Jin *et al.*, 2007b; Peng *et al.*, 1998).

The first function found for HCPro was assistance during aphid transmission (HC stands for helper component) (Atreya & Pirone, 1993). For viruses from *Potyvirus* genus, it

has been proposed that HCPro acts as a "bridge" between virion, through the interaction with CP (Blanc *et al.*, 1997; Peng *et al.*, 1998), and aphids, interacting with insect stylet (Wang *et al.*, 1998). Interestingly, it was also shown that HCPro is also implicated in mite transmission of tritimoviruses (Stenger *et al.*, 2006; Stenger *et al.*, 2005).

The HCPro was one of the first characterized RSS, and the interaction with double-stranded siRNAs was postulated as molecular mechanism of action (see section I.1.4).

In the case of bymoviruses, the equivalent products to P1 and HCPro are in a separate shorter RNA, but very limited information regarding the roles for P2-1 and P2-2 proteins is available, and their reduced similarity with other potyviral products makes it difficult to attribute functions. Transmembrane domains present in P2-2 have been hypothesized to play a role in vector transmission (Adams *et al.*, 2001).

- P3 and P3N+PIPO:

P3 has remained for years as one of the least characterized proteins within the potyviral genome, devoid of any clear function. Similarly to P1 protein, this factor is barely conserved among different potyviruses. It has been shown that TVMV P3 interacts with the cytoplasmic cylindrical inclusions (Rodríguez-Cerezo *et al.*, 1993), whereas that of TEV interacts with nuclear inclusions (Langenberg & Zhang, 1997). Moreover, using different heterologous systems, binding of potyviral P3s to P1, NIa and NIb have also been reported (Guo *et al.*, 2001; Merits *et al.*, 1999).

The discovery of the *pipo* ORF might help to explain some previous results about P3, facilitating the finding of any function for this factor. This new product, named P3N+PIPO (Fig. I3), is likely to be translated throughout a ribosomal frameshift, and its participation in one or some of the most basic viral functions is probably essential, since TuMV mutants altering this ORF were not infectious (Chung *et al.*, 2008). Moreover, recent results have shown that P3N+PIPO is required for potyvirus movement (Wei *et al.*, 2010a; Wen & Hajimorad, 2010). P3N+PIPO is a plasmodesmata-associated protein that interacts with CI modulating its targeting to this specialized intercellular organelle. Hence, the CI-P3N+PIPO complex could facilitate the cell-to-cell movement of potyviruses by promoting the formation of conical structures anchored to and extend through plasmodesmata (Wei *et al.*, 2010a).

- 6K1 and 6K2:

Two small 6K cistrons are present in the genome, flanking the CI region. The function of the first 6K1 cistron is not well known. This protein contains a hydrophobic region that could mediate its membrane integration. Sometimes 6K1 is even considered as part of a joint P3+6K1 protein given that processing *in vitro* between both proteins is only partial (García *et al.*, 1992). Although the cleavage of these viral proteins appear not to be essential for PPV viability, mutations affecting the efficiency of processing disturbed both the time course and severity of the induction of symptoms (Riechmann *et al.*, 1995). However, mature 6K1 product has been found in PPV infected cells, suggesting that this peptide could play a functional role by itself (Waltermann & Maiss, 2006).

The second small 6K2 peptide resembles 6K1 in having a central hydrophobic domain. It has been found that TEV 6K2 is associated with endoplasmic reticulum membranes via this domain; moreover, it has been suggested that this factor could be involved in RNA replication through a mechanism of retention of the replication complex in virus-induced membranous structures (Schaad *et al.*, 1997a). Results with PVA reveal roles for 6K2 in movement and symptom induction in a host-specific manner (Spetz & Valkonen, 2004).

- CI:

The CI protein is the largest potyviral gene product. It forms the very distinctive pinwheel-shaped cylindrical inclusions in the cytoplasm of infected cells with high taxonomic value because they are unique to members of the family *Potyviridae* (Fig. I3B). This viral factor is member of the large group of proteins with an NTP-binding domain, feature that is both widespread in nature and present in most of RNA viruses (Gorbalenya & Koonin, 1989; Laín *et al.*, 1989). CI exhibits ATPase and RNA helicase activities, and is supposed to act during RNA replication and perhaps in translation by helping to unwind the RNA (Fernández *et al.*, 1997).

Associations of CI with host factors have been reported (Bilgin *et al.*, 2003; Jiménez, 2004; Jiménez *et al.*, 2006); for instance, the interaction between PPV CI and *Nicotiana benthamiana* PSI-K (a protein from photosystem I) has been proposed as relevant given that down-regulation of this gene lead to higher viral accumulation (Jiménez *et al.*, 2006). CI appears to be also implicated in viral movement (Carrington *et al.*, 1998; Gómez de Cedrón *et al.*, 2006), a fact that agrees with its presence in structures near plasmodesmata (Roberts *et al.*, 1998; Rodríguez-Cerezo *et al.*, 1997). This specific localization of CI appears to depend on the P3N+PIPO protein (see above).

- NIa (VPg-Pro):

The NIa (**n**uclear **i**nclusion protein a) is one of the proteins that form the crystalline inclusions that accumulate in the nucleus of infected cells in many potyviral infections. The sequence coding for NIa comprises essentially two cistrons due to the presence of an internal suboptimal cleavage site (Schaad *et al.*, 1996). The N-proximal part constitutes the VPg, which is attached covalently to the 5'-end of the genomic RNA. The VPg is involved in replication possibly by priming the RNA synthesis by a similar mechanism as proposed for picornaviruses (Anindya *et al.*, 2005; Puustinen & Mäkinen, 2004). It is also implicated in long distance movement, and it has been associated with host range determination (Rajamäki & Valkonen, 1999; Schaad *et al.*, 1997b). Interaction between VPg and eukaryotic initiation factors points towards a central role in pathogenicity that might serve to explain some resistance mechanisms (Charron *et al.*, 2008).

The role of the NIa protein in the nucleus of virus-infected cells is largelly unknown. NIa is directed to the nucleus by a nuclear localization signal (NLS) located in the VPg domain (Carrington *et al.*, 1991; Rajamaki & Valkonen, 2009; Schaad *et al.*, 1996). In the case of PVA NIa, a bipartite NLS in the VPg is controlling not only the nuclear localization of NIa, but also its specific localization in different compartments of the nucleus (Rajamaki & Valkonen, 2009). The fact that NIa displays non-specific deoxyribonuclease activity, has prompted the suggestion that its presence in the nucleus might serve to degrade the host DNA (Anindya & Savithri, 2004).

The protease activity responsible for cleaving part of viral polyprotein resides in the C-termini of the NIa (Pro, Fig. I4A). This protease is highly specific in the recognition of its cleavage sites, which has made it an interesting tool for biotechnological applications.

- NIb:

NIb is the second component of the potyviral nuclear inclussions. NIb has homology to known RNA-dependent RNA polymerases. It displays RNA polymerase activity *in vitro*, being presumably the viral replicase (Hong & Hunt, 1996). Interactions with both viral and host-encoded factors could help to this NIb function (Hong *et al.*, 1995; Li *et al.*, 1997; Merits *et al.*, 1999; Wang *et al.*, 2000), but the relevance of them are still unknown.

The NIb is directed to the nucleus of infected cells by specific signals in its sequence, and its recruitment to the replication complexes is postulated to occur via interaction with NIa (Daròs *et al.*, 1999).

- CP:

Besides encapsidating the viral RNA, the CP is implicated in cell-to-cell movement (Dolja *et al.*, 1995; Rojas *et al.*, 1997), and also in aphid transmission through interaction with HCPro (Blanc *et al.*, 1997). The existence of host-dependent posttranslational modifications of the CP, such as phosphorylation and glycosylation, has been reported in some potyviruses (Chen *et al.*, 2005; Ivanov *et al.*, 2003). Additionally, CP has been shown to display NTPase activity, but the functional relevance of this activity is unknown (Rakitina *et al.*, 2005).

Given the high variability of the CP N-terminal region between potyviruses, it has been proposed that this part of the protein could mediate interactions with specific host factors of functional relevance. The importance of the N-terminus of CP for infectivity and transmissibility by aphids has been studied for some potyviruses (Arazi *et al.*, 2001; López-Moya & Pirone, 1998; López-Moya *et al.*, 1999). Interestingly, the determinants of potyvirus ability to overcome the RTM resistance, which restrict the vascular movement of potyviruses in *Arabidopsis thaliana*, maps to the CP N-terminus (Decroocq *et al.*, 2009).

It has been recently reported that PVA CP interacts with HSP70 and its co-chaperone CPIP. These interactions appear to be involved in complex processes that modulate viral translation and replication through the modification of CP stability (Hafren *et al.*, 2010).

I.3 Objectives

Initially, HCPro was considered the only protein of viruses from the *Potyviridae* family with the ability to counteract the RNA silencing antiviral defense barrier, and thus, it was assumed that this protein was essential for all potyvirids. Genome sequencing of a large number of members of the genera *Potyvirus*, *Rymovirus* and *Tritimovirus*, as well as of the type member of the genus *Ipomovirus* appeared to support this hypothesis. However, further sequencing of additional members of the genus *Ipomovirus* revealed an unexpected variability at the 5' terminal region, including the P1 and HCPro cistrons, of the genomic RNAs of viruses of this genus. The first evidence of this genetic divergence came from the sequencing of the genome of CVYV, which revealed the absence of a coding sequence for HCPro, whereas the CVYV genome appeared to contain an extraordinarily long P1 cistron. With these antecedents, the overall objective of this work was to identify the viral proteins encoded by the 5'-terminal region of the genomes of different members of the family *Potyviridae* and to determine their contribution to suppress the antiviral RNA silencing response of the plant. I was especially interested in understanding how highly related viruses depend on quite different factors to face similar antiviral challenges.

For this general goal, we raised the next specific objectives:

A- Inference of putative functions of potyvirid P1 proteins in the infection cycle from the analysis of their amino acid sequences using *in silico* approaches.

B- Identification of factors contributing to RNA silencing suppression of the HCPro-defective CVYV.

C- Identification of the molecular mechanisms used by the CVYV RSS(s) to counteract plant defenses based on RNA silencing. Comparison with those of the typical potyviral RSS HCPro.

D- Assessment of the relevance of RNA silencing suppression activity and its specificity in potyviral infection.

Materials and Methods

II. MATERIALS AND METHODS

II.1 Plants

Transient expressions of proteins by agro-infiltration were done in *Nicotiana benthamiana*, either wild type or transgenic line 16c expressing constitutively GFP (a gift of David Baulcombe). Viral infectivity assays were carried out in herbaceous species such as *N. benthamiana*, *N. clevelandii* and *Cucumis sativus* Albatroz RZ F1 (Rijk Zwaan Iberica, Almería, Spain).

Plants were grown in a greenhouse maintained at 16 hours of light with supplementary illumination and 19-23 ºC.

II.2 Virus and bacterial strains

C. sativus leaves infected with CVYY were obtained from the German Collection of Microorganisms and Cell Cultures (code PV- 0724).

pIC-PPV-NK-GFP, which derives from PPV-R isolate, has been previously described (Fernández-Fernández *et al.*, 2001).

Escherichia coli DH5α was used for cloning of plasmids. *Agrobacterium tumefaciens* C58C1 was used for transient expression of proteins, whereas *A. tumefaciens* GV3101 containing the helper plasmid pJIC SA_Rep was used for the same purpose in case of inoculations of PVX variants.

II.3 Agro-infiltration

N. benthamiana and *N. clevelandii* were infiltrated with *A. tumefaciens* carrying the indicated plasmids. Appropriate *Agrobacterium* cultures were sedimented by centrifugation at 5000 x g at room temperature for 10 minutes and re-suspended in the induction buffer (10 mM MES pH 5.6, 10 mM $MgCl_2$ and 0.15 mM acetosyringone). Cells were left in this medium for 3 h at room temperature. Mixes of indicated strains were then prepared (DO_{600}=0.5 of each strain) and applied with a syringe to the underside of 2-3 leaves of 4-week-old plants.

II.4 Inoculation of viruses

II.4.1 Biolistic inoculation

The Helios Gene Gun System (Bio-Rad, Hercules, CA, US) was used for biolistic inoculation. Microcarrier cartridges were prepared with 1.0 μm gold particles coated with the

different plasmids at a DNA loading ration of 2 µg/mg of gold and a microcarrier loading quantity of 0.5 mg/shooting, according to the manufacturer's instructions. Helium pressure of 7.5 bar was used. Each cartridge was shot twice onto one plant, making one shoot for each of the two inoculated leaves.

II.4.1 Inoculation by agro-infiltration

Viral inoculations by infiltration of *A. tumefaciens* carrying the indicated binary vectors were carried out as described (see II.3). These vectors contained the viral cDNA into the T-DNA sequence of pBIN19.

II.5 Preparation, manipulation and analysis of nucleic acids

II.5.1 DNA plasmid preparation

The purification of plasmids from *E. coli* was performed by alkaline lysis method (Sambrook *et al.*, 1989). The preparation of plasmids from *A. tumefaciens* was done using the Wizard kit (Promega, Madison, WI, US).

II.5.2 DNA cloning

For digestion/ligation strategy, the DNA treatments with restriction enzymes (different commercial sources) and with T4 DNA ligase (Fermentas INC, Ontario, Canada) were carried out following the instructions of manufacturers. GATEWAY technology was also used, to construct plasmids, according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, US).

II.5.3 DNA amplification by PCR

DNA amplifications were carried out by the polymerase chain reaction (PCR) in a thermo-cycler PTC-100 (MJ Research Inc, Waltham, MA), using Expand High Fidelity (Roche, Basel, Switzerland) for PCRs including mutagenic primers, and Pwo (Roche, Basel, Switzerland) for the rest of reactions.

II.5.4 DNA electrophoresis in agarose gel and extraction

DNA fragments were separated by electrophoresis in 0.8% to 1.2% agarose gels, depending on the expected sizes, using 0.5X TBE (45 mM Tris-borate, 1 mM EDTA) as running buffer. The gels were prepared in presence of 0.1 mg/ml of BrEt in order to visualize

the DNA under UV light. Pictures of gels were taken in a Gel Doc 2000 image capturer (Bio-Rad, Hercules, CA, US).

QIAEX II system (QIAGEN, Hilden, Germany) was used to extract DNA fragment from agarose gels.

II.5.5 Preparation of large and small RNAs

Total RNA was isolated from agroinfiltrated leaf tissue by the method described by Lagrimini *et al*. (1990). After LiCl precipitation, the pellet fraction containing high molecular weight RNAs, including mRNAs, was resuspended in water. The supernatant fraction containing low molecular weight RNAs was precipitated with ethanol and resuspended in water.

For purifications of small RNAs interacting with P1b *in vivo*, wild type and mutant NTAP-P1b were purified by affinity chromatography in calmoduline-Sepharose beads (see II.7.3). Protein fractions were mixed with one-half volume of RNA extraction buffer (100 mM Tris-HCl pH 8, 100 mM LiCl, 10 mM EDTA and 1% SDS) and one-half volume of phenol. The nucleic acids were then precipitated from the aqueous phase with ethanol, using GlycoBlue (Ambion, Austin, TX, US) as carrier, and resuspended in water.

II.5.6 Northern blot analysis

For Northern blot analysis of GFP mRNA, fractions of high molecular weight RNAs (10 μg) were separated on a 1.2% agarose gel containing 6% formaldehyde, using 1X MOPS as running buffer, and transferred to a Nylon Zeta-Probe membrane (Bio-Rad, Hercules, CA, US) by capillary blotting. After UV cross-linking and prehybridization in UltraHyb (Ambion, Austin, TX, US), blots were hybridized in the same solution with ^{32}P-labeled DNA probes specific to the GFP coding sequence, synthesized with Rediprime II Random Prime Labeling System (GE Healthcare, Buckinghamshire, UK). BrEt staining of the gel was used to verify equal loading.

For Northern blot analysis of GFP siRNAs, fractions of low molecular weight RNAs were resolved on a 15% polyacrylamide gel containing 7M urea, and transferred to a Nylon Zeta-Probe membrane using a transblot semidry transfer cell (Bio-Rad, Hercules, CA, US). After UV cross-linking and prehybridization in UltraHyb (Ambion, Austin, TX, US), blots were hybridized in the same solution with specific probes. siRNAs generated from different parts of GFP RNA were detected with ^{32}P-labeled antisense GFP, GF and P riboprobes, which were prepared by transcription with SP6 RNA polymerase (Roche, Basel, Switzerland) from

*BamH*I-linealized pGemT-GFP, *SacII*-linearized pGemT-GF and pGemT-P, respectively. These plasmids contain the nt 1 to 717 (GFP), nt 4 to 403 (GF) and nt 404 to 717 (P) of the GFP gene cloned into pGemT. After transcription reaction, riboprobes were fragmented by hydrolysis with 15 volumes of carbonate buffer (80 mM $NaHCO_3$, 120 mM $NaCO_3$) to an average length of 50 nt (Hamilton and Baulcombe, 1999). BrEt staining of gels was used to verify equal loading.

Both GFP mRNA and GFP siRNA hybridization signals were detected with a Molecular Imager FX system (Bio-Rad, Hercules, CA, US).

II.5.7 High-throughput sequencing of small RNAs

Production of small RNA libraries was done according to Mosher *et al*. (2009). Total leaf RNA (100 µg) was run over miRVana kit (Ambion, Austin, TX, US) to enrich the samples in RNAs shorter than 200-nt, whereas this step was omitted for nucleic acids that were co-purified with NTAP-P1b.

Purified libraries were sequenced on an Illumina Genome Analyser (Illumina, San Diego, CA, US) and the reads were processed by Sequence Pre-processing, Filter, and miRProf tools (http://srna-tools.cmp.uea.ac.uk, Moxon *et al.*, 2008) to remove the 3' adaptor, discard known tRNA and rRNA molecules, and detect conserved miRNAs, respectively. Identification of reads matching the pNTAP-P1b plasmid sequence and treatment of the data to present them as histograms were carried out using in-house developed PHP scripts.

II.5.8 Electrophoretic mobility shift assay (EMSA)

Synthetic nucleic acids (Sigma, St. Louis, MO, US) used for EMSA are listed in Table M1. They were labelled with [γ-^{32}P]ATP by using T4 polynucleotide kinase (PNK, Promega, Madison, WI, US). Non-labelled 5'-phosphorylated siRNAs were also obtained by PNK treatment using ATP as phosphate group donor.

Crude protein extracts for binding reactions were prepared by homogenizing agro-infiltrated tissue powdered under liquid nitrogen in binding buffer (83 mM Tris-HCl, pH 7.5, 0.8 mM MgCl2, 66 mM KCl, 100 mM NaCl, 2 mM ß-mercaptoethanol) (4 ml/mg) and clarified by centrifugation at 18000 x g at 4°C for 15 min.

^{32}P siRNAs (0.5 nM) were incubated, in presence or absence of non-labeled competitor, for 30 min at room temperature with different amounts of either affinity chromatography-purified NTAP-P1b proteins or crude protein extracts from agroinfiltrated tissue, in a reaction mixture (20 µl) containing binding buffer and 16U of RNAse inhibitor (Takara Bio Inc., Otsu,

Shiga, Japan). After incubation, protein-RNA complexes were resolved on 5% polyacrylamide-containing 0.5 X TBE gels. The gels were dried and exposed to X-ray sensitive films. For super shift assays, variable amounts of PAP complex (Sigma-Aldrich, St. Louis, MO, US) were included in the binding mixtures.

II.6 Construction of plasmids

II.6.1 Plasmids for transient expression by agroinfiltration

To obtain a plasmid carrying the cDNA sequence of the 5'-terminal part of the CVYV genome, the 5' UTR, except the first 6 nt, plus the P1 coding sequence were amplified by two RT-PCRs using as template a crude nucleic acid extract from infected cucumber leaves. Primers #821 and #824 (Table M3) were used in one of the reactions to amplify a fragment including CVYV nt 7-1667 (numbering is according to Jansen *et al*. (2005). This fragment was cloned into pCRII by the TOPO-cloning system (Invitrogen, Carlsbad, CA, US) to create pCRII-5'P1. The second RT-PCR used primers #823 and #822 (Table M3) to amplify the CVYV cDNA fragment 1066-2596, which was digested with *Sal*I and cloned into pUC19 digested with *SmaI* and *SalI,* to create pUC-3'P1. pUC-P1$_{CVYV}$, which includes the assembled CVYV 5'UTR-P1 coding sequence, was obtained by cloning into pUC19 digested with *EcoR*I and *Kpn*I, the *EcoR*I/*Sal*I and *Sal*I/*Kpn*I fragments from pCRII-5'P1 and pUC-3'P1, respectively, including the partial CVYV sequences. The CVYV insert of pUC-P1$_{CVYV}$ was sequenced (GeneBank accession number DQ496114); this has 2590 nt and 19 nt changes compared to the sequence published by Jansen *et al.* (2005).

GATEWAY technology (Invitrogen, Carlsbad, CA, US) was applied to construct plasmids expressing proteins from the N-terminal region of either PPV or CVYV polyproteins, using pDONR-207 (Invitrogen) as donor vector and pDEST-TH1 (provided by Helena Berglund, Karolinska Institutet, Stockholm) (Hammarström *et al.*, 2002), pGWB2 (provided by Tsuyoshi Nakagawa, University of Shimane) (Nakagawa *et al.*, 2007) pNTAPi and pCTAPi (provided by Michael Fromm, University of Nebraska) (Rohila *et al.*, 2004), pBIFP2 and pBIFP3 (provided by François Parcy, Laboratoire Physiologie Cellulaire Végétale, Grenoble) (Desprez *et al.*, 2007).

Site-directed mutagenesis of P1b was carried out by two PCR steps as described by Herlitze and Koenen (1990). Primers and templates used for PCR amplifications of viral

sequences, including the site-directed mutagenesis of P1b, to generate the entry vectors are listed in Tables M2 and M3.

Expression vectors producing PPV 5'UTR-P1-HCPro (p35S-P1HC), CVYV 5'UTR-P1 (p35S-P1), 5'UTR-P1a (p35S-P1a) and P1b (p35S-P1b), including termination codons at the end of sequences in all cases, were constructed by LR clonase reactions between the destination vector pGWB2 and pDONR-5'P1HC, pDONR-5'P1, pDONR-5'P1a and pDONR-AUGP1b entry vectors, respectively.

Expression vectors producing CVYV P1-CTAP (p35S-P1$_{CVYV}$-CTAP) and P1b-CTAP (p35S-P1b$_{CVYV}$-CTAP) lacking the last codon of the P1b sequence to avoid proteolytic processing of the recombinant product by P1b action, were constructed by LR clonase reactions between the destination vector pCTAPi and pDONR-5'P1noncut and pDONR-P1bnoncut entry vectors, respectively.

Expression vectors producing wild type and mutant P1b-CTAP (p35S-P1b-CTAP and its derivatives) carrying the sequence of the initial amino acid of CVYV P3 at the end of the P1b sequence to permit the proteolytic processing of the recombinant product by P1b action, were constructed by LR clonase reactions between the destination vector pCTAPi and pDONR-P1bcut entry vectors.

Expression vectors producing wild type and mutant NTAP-P1b (p35S-NTAP-P1b and its derivatives), NYFP-P1b (p35S-NYFP-P1b and its derivatives), CYFP-P1b (p35S-CYFP-P1b and its derivatives), and MBP-P1b (pMBP-P1b) were constructed by LR clonase reactions between pDONR-nonAUGP1b entry vectors and the destination vectors pNTAPi, pBIFP2, pBIFP3, and pDEST-TH1, respectively.

A. tumefaciens C58C1 strain carrying p35S:GFP (Haseloff *et al.*, 1997) plus pCH32 (Hamilton *et al.*, 1996), *A. tumefaciens* GV3101 strain carrying pJIC_SA Rep, and both p35S:GF-IR (Schwach *et al.*, 2005) and pBIN61:P19 (Voinnet *et al.*, 2003) plasmids were kindly provided by David Baulcombe (University of Cambridge, United Kingdom).

II.6.2 Plasmids for viral infection

A partial PPV-R clone (p35SeNOSB) carrying the cDNA corresponding to the 5' region of the viral genome (nucleotides 1-3628) cloned between the CaMV 35S promoter and the NOS terminator (López-Moya & García, 2000), in which the first AUG of the large ORF was mutated and the second AUG was engineered to display an *Nco*I restriction site (Simón-Buela *et al.*, 1997), was used as backbone to generate intermediate cDNA clones corresponding to the 5' region of the PPV-R genome either solely lacking HCPro (p35S-P1ΔHC) or with wild

type or mutant versions of the CVYV P1b gene replacing the HCPro sequence (p35S-P1P1b and derivatives). The gene splicing via overlap extension method (Horton *et al.*, 1989) was used to generate these constructs. Primers and templates for PCRs used to make these viral cDNA clones are listed in Tables M4 and M5.

p35S-P1ΔHC was obtained by replacing the *Nco*I-*Dra*III fragment of p35SeNOSB that encodes P1-HCPro-P3, with the corresponding fragment from PCR3, which codes for PPV P1-P3 and lacks the HCPro coding sequence. The p35S-P1P1b clone was obtained by inserting a *Nco*I-*BamH*I fragment from PCR6 and a *BamH*I-*Dra*III fragment from PCR9, which together code for PPV P1- CVYV P1b-PPV P3 in p35SeNOSB digested with *Nco*I and *Dra*III. p35S-P1P1b clones carrying RK68,69AA, C89A or C93A mutant versions of CVYV P1b were obtained by replacing the *Nco*I-*BamH*I fragment from p35S-P1P1b that codes for P1-P1b, with the corresponding fragment from PCR11, PCR13 and PCR14, respectively.

Full-length clones of P1ΔHC and P1P1b chimerical viruses (Fig. R34B) were constructed by replacing the *Xba*I-*Dra*III fragment of pIC-PPV-NK-GFP that contains the sequence of the 35S promoter followed by the cDNA sequence of the PPV 5'UTR plus the sequence encoding PPV P1-HCPro-P3, with the corresponding fragments from p35S-P1ΔHC and p35S-P1P1b (carrying wild type or mutants versions of CVYV P1b), respectively. Note that this cloning strategy renders P1P1b chimerical viruses coding for PPV P1 and CVYV P1b fused to the two first amino acids of PPV HCPro and CVYV P3, respectively, to allow that both serine proteases cut efficiently at their carboxi ends.

GATEWAY technology (Invitrogen, Carlsbad, CA, US) was also applied to construct plasmids expressing PVX chimera carrying wild type and mutant versions of CVYV P1b. These plasmids were obtained by LR clonase reactions between pDONR-P1bcut entry vectors (Table M2) and the destination vector pGWC-PVX (a GATEWAY-adapted plasmid derived from pGR106, García *et al*., manuscript in preparation).

II.7 Protein analysis

II.7.1 Western blot analysis

Agro-infiltrated and infected tissues (or equivalent tissues from mock inoculated plants) were ground to a fine powder under liquid nitrogen and stored at –80ºC until use. Protein extracts were prepared by thawing the powder in extraction buffer (150 mM Tris-HCl pH 7.5, 6 M Urea, 2% SDS and 5% ß-mercaptoethanol) (2 ml/mg). Samples were boiled for 5 minutes and cell debris removed by centrifugation at 18000 x g at 4ºC for 10 minutes.

Supernatants were resolved on SDS-PAGE (12% acrylamide), electroblotted to a nitrocellulose membrane and subjected to immunodetection.

Specific proteins were detected using the TAP-reactive PAP soluble complex (Sigma-Aldrich, St. Louis, MO, US), or the following combinations of primary and secondary reagents: anti-HCPro, anti-P1b or anti-CP rabbit sera, with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Jackson, West Grove, PA, US), a mixture of two anti-GFP monoclonal antibodies (Roche, Basel, Switzerland) with HRP-conjugated sheep anti mouse IgG (Sigma-Aldrich, St. Louis, MO, US), or biotinylated calmoduline (Calbiochem, Nottingham, UK) with streptavidin-HRP (GE Healthcare, Buckinghamshire, UK). The immunostained proteins were visualized by enhanced chemiluminescence detection with a LifeABlot kit (EuroClone, Siziano, Italy) according to the manufacturer's instructions. Ponceau red staining was used to check the global protein content of the samples.

II.7.2 Purification of recombinant MBP-P1b and production of anti-P1b sera

E. coli DH5α transformed with pMBP-P1b was used to produce CVYV P1b fused to MBP following a protocol previously described for MBP-tagged CI protein (Fernández *et al.*, 1995), except that bacteria were lysed by sonication. MBP-P1b (500 µg) was emulsified in Freund's complete (for initial immunization) or incomplete (for three subsequent boosters) Freund's adjuvant, and injected into rabbits. Serum was collected 15 days after the last booster and stored at -20º.

II.7.3 Purification of TAP-tagged P1b proteins

Patches of *N. benthamiana* leaves infiltrated with *A. tumefaciens* carrying p35S-NTAP-P1b plasmids were harvested at 6 days post infiltration (dpi), ground to a fine powder under liquid nitrogen and stored at -80°C until use. This powder was incubated with extraction buffer consisting of 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 2 mM $Mg(CH_3COO)_2$, 4 mM $CaCl_2$, 2 mM ß-mercaptoethanol, 0.1% NP-40, 10 µM leupeptin, 1 µM pepstatin, 1 µM aprotinin, 1 mM PMSF, 10 µM E-64 (2 mg/ml) by 30 minutes at 4ºC, and cell debris were removed by centrifugation at 18000 x *g* at 4°C for 10 minutes. The supernatant was diluted ten times with extraction buffer lacking the protease inhibitors, filtered through a 0.45 µm nitrocellulose membrane (Millipore, Billerica, MA, US) and loaded in an Econo-column (Bio-Rad, Hercules, CA, US) packed with 1 ml of calmoduline-sepharose beads (GE Healthcare, Buckinghamshire, UK). After washing with 20 ml of a buffer containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, the bound proteins were eluted with washing buffer supplied with 2

mM EGTA. TAP removal in the purified proteins was carried out with AcTEV protease (Invitrogen, Carlsbad, CA, US) according to the manufacturer's instructions.

II.7.4 Gel filtration-FPLC

Affinity-purified TAP-tagged P1b proteins, intact or proteolytically processed by AcTEV protease, were analyzed by gel filtration using a fast protein liquid chromatography system (ÄKTA-Prime, GE Healthcare, Buckinghamshire, UK) with a Hi-load 16/60 Superdex-200 column (GE Healthcare, Buckinghamshire, UK) at 4°C. Column equilibration and chromatography were performed at a flow rate of 0.5 ml/min in a buffer consisting of 20 mM Tris-HCl pH 7.5, 150 mM NaCl. Fractions were collected every 2.5 ml and subjected to Western blot assays.

The column was calibrated with catalase (158 kDa), serum albumin (68 kDa), ovoalbumine (50 kDa) and chymotrypsinogen A (21 kDa), using the same chromatography protocol.

II.7.5 Fluorescence observation and imaging

GFP fluorescence was monitored either by visual inspection under long-wavelength UV light (Black Ray model B 100 AP; Ultra-Violet Products, Upland, CA, US) or under a Leica MZ FLIII epifluorescence stereomicroscope (Leica, Wetzlar, Germany) with excitation and barrier filters of 480/40 and 510 nm.

For YFP observation in Bimolecular Fluorescence Complementation assays, little pieces of agroinfiltrated leaves were examined with a Leica DMR epifluorescence microscope using excitation and barrier filters at 450/90 nm and 500/550 nm, respectively.

Pictures were caught with a Nikon D1X digital camera (Nikon, Chiyoda-ku, Tokyo, Japan) equipped with a black-and-white 62E 022 filter, and with an Olympus DP 70 camera coupled to either microscope using DP Controller and DP manager software (Olympus Optical, Tokyo, Japan).

II.7.6 Analysis of sequences in silico

Table M6 lists monopartite members of the family *Potyviridae* considered in this work. In most cases, a single sequence for each virus species was used for the analyses (labelled in bold). The EditSeq program of the DNASTAR software suite, as well as online facilities at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov) was used for BLAST similarity searches. The EditSeq program was also used for isoelectric point

calculations. Plots of charge density at pH 7 were made with the DNASTAR Protean program with a window size of five residues.

An automated system for phylogenetic detection of recombination using a genetic algorithm (Kosakovsky Pond *et al.*, 2006) known as GARD (Genetic Algorithm for Recombination Detection) and available at http://www.datamonkey.org was used to search for evidence of recombination breakpoints in selected nucleotide alignments of P1 sequences.

Table M1. Sequence of synthetic nucleic acids used in EMSA assays.

Description	Sequence (5'-3')
21-nt ssRNA with 2-deoxinucleotide at the 3'end	5'- CUUACGCUGAGUACUUCGATT -3'
21-nt dsRNA with 2-deoxinucleotide 3' overhangs	5'- CUUACGCUGAGUACUUCGATT -3' and 5'- UCGAAGUACUCAGCGUAAGTT -3'
24-nt dsRNA with 2-deoxinucleotide 3' overhangs	5'- CACUUACGCUGAGUACUUCGGATT -3' and 5'- UCCGAAGUACUCAGCGUAAGUGTT -3'
26-nt dsRNA with 2-deoxinucleotide 3' overhangs	5'- UCACUUACGCUGAGUACUUCGAGATT -3' and 5'- UCUCGAAGUACUCAGCGUAAGUGATT -3'
19-nt dsRNA blund end	5'- CUUACGCUGAGUACUUCGA -3' and 5'- UCGAAGUACUCAGCGUAAG -3'
21-nt ssDNA	5'- CTTACGCTGAGTACTTCGATT -3'
21-nt dsDNA with 2-deoxinucleotide 3' overhangs	5'- CTTACGCTGAGTACTTCGATT -3' and 5'- TCGAAGTACTCAGCGTAAGTT -3'

Table M2. List of primers and templates used for PCR reactions in the construction of the different entry vectors.

Entry plasmid	Forward [a]	Reverse [a]	Template
1- pDONR-5'P1HC	#697	#820	pIC-PPV-NK-GFP
2- pDONR-5'P1	#821	#822	pUC-P1
3- pDONR-5'P1a	#821	#837	pUC-P1
4- pDONR-AUGP1b	#835	#822	pUC-P1
5- pDONR-5'P1noncut	#821	#836	pUC-P1
6- pDONR-P1bnoncut	#835	#836	pUC-P1
7- pDONR-nonAUGP1b	#838	#822	p35S-P1b
8- pDONR-nonAUGP1b RK68,69AA			
Mutagenic PCR	#839	#822	p35S-P1b
2nd PCR	#838	#822	1st PCR product + A[b]
9- pDONR-nonAUGP1b C89A			
Mutagenic PCR	#842	#822	p35S-P1b
2nd PCR	#838	#822	1st PCR product + A[b]
10- pDONR-nonAUGP1b C93A			
Mutagenic PCR	#843	#822	p35S-P1b
2nd PCR	#838	#822	1st PCR product + A[b]
11- pDONR-nonAUGP1b C103A			
Mutagenic PCR	#844	#822	p35S-P1b
2nd PCR	#838	#822	1st PCR product + A[b]
12- pDONR-nonAUGP1b C106A			
Mutagenic PCR	#845	#822	p35S-P1b
2nd PCR	#838	#822	1st PCR product + A[b]
13- pDONR-nonAUGP1b H221A			
Mutagenic PCR	#838	#847	p35S-P1b
2nd PCR	#838	#822	1st PCR product + B[b]
14- pDONR-nonAUGP1b S264A			
Mutagenic PCR	#838	#848	p35S-P1b
2nd PCR	#838	#822	1st PCR product + B[b]
15- pDONR-nonAUGP1b KR10,11AA			
Mutagenic PCR	#964	#822	pNTAP-P1b
2nd PCR	#838	#822	1º PCR plus DNA fragment [b]
16- pDONR-nonAUGP1b K61A			
Mutagenic PCR	#966	#822	pNTAP-P1b
2nd PCR	#838	#822	1º PCR plus DNA fragment [b]
17- pDONR-nonAUGP1b R68L			
Mutagenic PCR	#968	#822	pNTAP-P1b
2nd PCR	#838	#822	1º PCR plus DNA fragment [b]
18- pDONR-nonAUGP1b R68A			
Mutagenic PCR	#1110	#822	pNTAP-P1b
2nd PCR	#838	#822	1º PCR plus DNA fragment [b]
19- pDONR-nonAUGP1b K69A			
Mutagenic PCR	#969	#822	pNTAP-P1b

2nd PCR	#838	#822	1º PCR plus DNA fragment [b]
20- pDONR-P1bcut	#835	#890	pDON-nonAUGP1b
21- pDONR-P1bcut RK68,69AA	#835	#890	pDONR-nonAUGP1b RK68,69AA
22- pDONR-P1bcut C89A	#835	#890	pDONR-nonAUGP1b C89A
23- pDONR-P1bcut H221A	#835	#890	pDONR-nonAUGP1b H221A
24- pDONR-P1bcut S264A	#835	#890	pDONR-nonAUGP1b S264A

[a] The sequences of the primers are shown in Table 2

[b] A, HindIII-NheI fragment from pDONR-nonAUGP1b; B, PstI fragment from pDONR-nonAUGP1b.

Table M3. Sequences of PCR and RT-PCR primers used in the construction of entry vectors.

Primer	Sequence (5'-3')
#697	ggggacaagtttgtacaaaaaagcaggctGGAAAATATAAAAACTCAACACAAC
#820	ggggaccactttgtacaagaaagctgggTTATCCAACCAGGTATGTTTTCATATT
#821	ggggacaagtttgtacaaaaaagcaggctCAAAACATTCGATCACATATTATAAC
#822	ggggaccactttgtacaagaaagctgggTTAATAAAAGTCAATTTTATCTTTCTC
#823	GCATGGAAATGTGAGG
#824	TGAACGCATGCAATCC
#835	ggggacaagtttgtacaaaaaagcaggctCCATGGCAACAATTCATGGATTGCATGCGTTC
#836	ggggaccactttgtacaagaaagctgggtcAAAGTCAATTTTATCTTTCTCATC
#837	ggggaccactttgtacaagaaagctgggTTAATTGCGAATCCTTCTTAATTGTGAC
#838	ggggacaagtttgtacaaaaaagcaggctTCACAATTCATGGATTGCATGCG
#839	TTGGCATGCTTG**GC**A**GC**TGCAACTAAGTATGG
#842	GTTTGTTGCAGA**GC**TCACGTGAGATGC
#843	TGTCACGTGAGA**GC**CACTGATATATC
#844	ATGAATACCATG**GC**CAACGAGTGTGGG
#845	ATGTGCAACGAG**GCC**GGGGAGAACATGTTTG
#847	CAACCCAAAGAT**CGC**GTTCAATGTAATAC
#848	TATGACTACCCC**GGC**CCATCCTGGGC
#890	ggggaccactttgtacaagaaagctgggTCACAATAAAAGTCAATTTTATCTTTCTCATCTGC
#964	CATGCGTTCAAG**GCCGC**ATATCAAACGAACG
#966	AGTGGCGTCACA**GC**ACCAGCTTTGGCATGC
#968	TTGGCATGCTTG**CTT**AAGGCAACTAAGTATGG
#969	GCATGCTTG**C**G**CGC**GGCAACTAAGTATGG
#1110	TTGGCATGCTTG**GCC**AAGGCAACTAAGTATGG

GATEWAY recombination sequences and restriction sites needed for cloning purposes are indicated in lower case and mutated residues are in bold.

Table M4. List of primers and templates used for PCR reactions in the construction of PPV-derived recombinant viruses.

Plasmid	Forward [a]	Reverse [a]	Template
1- p35S-P1ΔHC			
PCR1	#976	#1074	p35SeNOSB
PCR2	#1075	#981	p35SeNOSB
PCR3	#976	#981	PCR1 + PCR2
2- p35S-P1P1b			
PCR4	#976	#977	p35SeNOSB
PCR5	#978	#979	pDONR-P1bcut
PCR6	#976	#979	PCR4 + PCR5
PCR7	#980	#981	p35SeNOSB
PCR8	#982	#983	pDONR-P1bcut
PCR9	#982	#981	PCR7 + PCR8
3- p35S-P1P1b RK68,69AA			
PCR10	#978	#979	pDONR-nonAUGP1b RK68,69AA
PCR11	#976	#979	PCR4 + PCR10
4- p35S-P1P1b C89A			
PCR12	#978	#979	pDONR-nonAUGP1b C89A
PCR13	#976	#979	PCR4 + PCR12
5- p35S-P1P1b C93A			
PCR14	#978	#979	pDONR-nonAUGP1b C93A
PCR15	#976	#979	PCR4 + PCR12

[a] The sequences of primers are shown in Table M5.

Table M5. Sequences of PCR primers used in the construction of PPV-derived recombinant viruses.

Primer	Sequence (5'-3') [a]
#976	gtcaccATGGCAACCATTG
#977	*CGCATGCAATCCATGAATTGT*GTCAGAGTAGTGGATTATCTC
#978	GAGATAATCCACTACTCTGAC*ACAATTCATGGATTGCATGCG*
#979	ccaaccaggt*AGAAGCAATAAAAGTCAATTTTATCTTTCTC*
#980	*GATAAAATTGACTTTTATTGCTTC*GGTCTTGAAGTGGATAAGTGTGACG
#981	CAAGCTTGCTCCAATTCCTGG
#982	*ACATTGAACCACATCTTTGGG*
#983	ACACTTATCCACTTCAAGACC*GAAGCAATAAAAGTCAATTTTATCTTTCTC*
#1074	GTCAGAGTAGTGGATTATCTCATTGC
#1075	AGCAATGAGATAATCCACTACTCTGACGGTCTTGAAGTGGATAAGTGTGACG

[a] Sequences corresponding to PPV are in plain upper case, sequences corresponding to CVYV are in italic upper case, restriction sites used for cloning are underlined.

Table M6. Virus species belonging to monopartite genera in the family *Potyviridae.*

Genus	Species	Acronym	Accession numbers*
Ipomovirus	*Cassava brown streak virus*	CBSV	**FJ039520**
	Cucumber vein yellowing virus	CVYV	**AY578085**
	Squash vein yellowing virus	SqVYV	**EU259611**
	Sweet potato mild mottle virus	SPMMV	**Z73124**
Potyvirus	*Bean common mosaic virus*	BCMV, Y-type isolates	**U34972**, U05771, AY968604, AJ312438, AY112735
		BCMV, R-type isolates	**AJ312437**, AY575773, AY863025
	Bean common mosaic necrosis virus	BCMNV NL-3 K	**AY864314**
		BCMNV NL-3 D-type isolates	AY138897, **AY282577**, U19287
	Beet mosaic virus	BtMV	**AY206394**
	Bean yellow mosaic virus	BYMV	AY192568, **D83749**, U47033
	Chilli veinal mottle virus	ChiVMV	**AJ237843**
	Clover yellow vein virus	ClYVV	**AB011819**
	Cocksfoot streak virus	CSV	**AF499738**
	Cowpea aphid-borne mosaic virus	CABMV	**AF348210**
	Daphne mosaic virus	DapMV	**DQ299908**
	Dasheen mosaic virus	DsMV	**AJ298033**
	East asian passiflora virus	EAPV	**AB246773**
	Johnsongrass mosaic virus	JGMV	**Z26920**
	Japanese yam mosaic virus	JYMV	AB016500, **AB027007**
	Konjak mosaic virus	KoMV	**AB219545**
	Lily mottle virus	LMoV	**AJ564636**, AM048875
	Lettuce mosaic virus	LMV	**AJ278854**, AJ306288, X97704-5
	Leek yellow stripe virus	LYSV	**AJ307057**, AB194621-3
	Maize dwarf mosaic virus	MDMV	**AJ001691**, AM110758
	Onion yellow dwarf virus	OYDV	**AJ510223**
	Peanut mottle virus	PeMoV	**AF023848**
	Pennisetum mosaic virus	PeMV	**AY642590**
	Pepper mottle virus	PepMoV	AB126033, AF501591, **M96425**
	Papaya leaf distortion mosaic virus	PLDMV	**AB088221**
	Plum pox virus	PPV	AF401295-6, AJ243957, AM157175, AY184478, AY912055-8, AY953261-7, AY028309, **D13751**, DQ465242-3, M92280, X16415, X81083, Y09851
	Papaya ringspot virus	PRSV	AY010722, AY027810, AY162218, AY231130, DQ340769-71, DQ374152-3, **S46722**, X67673, X97251
	Pea seed-borne mosaic virus	PSbMV	AJ252242, **D10930**, X89997
	Peru tomato mosaic virus	PTV	**AJ437280**, AJ516010
	Potato virus A	PVA	AF533212, AF543709, AJ131400-3, **AJ296311**, Z21670
	Potato virus V	PVV	**AJ243766**
	Potato virus Y	PVY	A08776, AF237963, AF463399, AF522296, AJ439544-5, AJ585342, AJ889866-8, AJ890342-50, AY166866-7, AY745491-2, AY884982-5, D00441, DQ008213, DQ157178-9, DQ309028, M95491, U09509, **X12456**, X97895
	Scallion mosaic virus	ScaMV	**AJ316084**
	Shallot yellow stripe virus	SYSV	**AJ865076**
	Sugarcane mosaic virus	SCMV	AF494510, AJ278405, AJ297628, AJ310102-5, AM110759, **AY042184**, AY149118, AY569692

	Soybean mosaic virus	SMV	AB100442-3, AF241739, AJ310200, **AJ312439**, **AJ507388**, AJ619757, AJ628750, AY216010, AY216987, AY294044-5, D00507, S42280
	Sweet potato feathery mottle virus	SPFMV	**D86371**
	Sorghum mosaic virus	SrMV	AJ310197, **AJ310198**, U57358
	Tobacco etch virus	TEV	L38714, **M11458**, M15239
	Thunberg fritillary virus	TFMV	**AJ851866**, AJ885005
	Turnip mosaic virus	TuMV	AB093596-627, AB105134-5, AB194785-802, **AF169561**, AF394601-2, AF530055, AY090660, AY134473, AY227024, D10927, D83184.
	Tobacco vein mottling virus	TVMV	**U38621**, X04083
	Watermelon mosaic virus	WMV	AB218280, **AY437609**, DQ399708
	Wild potato mosaic virus	WPMV	**AJ437279**
	Wisteria vein mosaic virus	WVMV	**AY656816**
	Yam mosaic virus	YMV	**U42596**
	Zucchini yellow mosaic virus	ZYMV	AB188115-6, AF014811, **AF127929**, AJ307036, AJ316228-9, AJ515911, AY188994, AY278998-9, AY279000, L29569, L31350
Rymovirus	*Agropiron mosaic virus*	AgMV	**AY623626**
	Hordeum mosaic virus	HoMV	**AY623627**
	Ryegrass mosaic virus	RGMV	**AF035818**, Y09854
Tritimovirus	*Brome streak mosaic virus*	BStMV	**Z48506**
	Oat necrotic mottle virus	ONMV	**AY377938**
	Wheat Eqlid mosaic virus	WeqMV	**EF608612**
	Wheat streak mosaic virus	WSMV	**AF057533**, AF285169-70, AF454454-5
Unclassified	*Blackberry virus Y*	BLVY	**AY994084**
	Sugarcane streak mosaic virus	SCSMV	**GQ388116**
	Triticum mosaic virus	TriMV	**FJ263671,** FJ669487

* Accession numbers for sequences used in the present study are highlighted in bold

Results

III. RESULTS

III.1 Recombination and gene duplication in the evolutionary diversification of P1 proteins in the family *Potyviridae*[*3]

Overall similarity along the complete *Potyviridae* polyprotein was rather high, with levels of amino acid identity of about 42-56% among different species of the same genus and of about 25-33% among viruses of different genera (Adams *et al.*, 2005b). However, conservation of individual mature proteins was very variable, being the first protein of the polyprotein, P1, the most divergent one, both in length and amino acid sequence (Adams *et al.*, 2005b).

Recombination is one of the main forces driving plant virus evolution, (García-Arenal *et al.*, 2003; Roossinck, 2003). Although frequent in many virus groups, recombination events are especially common in potyviruses (Chare & Holmes, 2006). Indeed, both intraspecies (Bousalem *et al.*, 2000; Cervera *et al.*, 1993; Glais *et al.*, 2002; Glasa *et al.*, 2004; Krause-Sakate *et al.*, 2004; Moreno *et al.*, 2004; Tan *et al.*, 2004; Zhong *et al.*, 2005) and interspecies (Ali *et al.*, 2006; Desbiez & Lecoq, 2004; Larsen *et al.*, 2005) recombination events are involved in potyviral evolution, some of which affect the P1 sequence (Ali *et al.*, 2006; Desbiez & Lecoq, 2004; Glais *et al.*, 2002; Larsen *et al.*, 2005; Tan *et al.*, 2004).

In this chapter we performed an extensive computational analysis of P1 proteins from 53 virus species of four *Potyviridae* genera. Our results suggest that not only intraspecies, but also intergenera recombination within the P1 gene contributed to *Potyviridae* evolution. P1 gene duplication is also shown to contribute to P1 diversification.

III.1.1 General and specific features of potyviral P1 proteins

The P1 gene, together with the coding sequence for the N-proximal region of the CP, shows the greatest variability in size and sequence within the potyvirus genome (Adams *et al.*, 2005b). However, well-conserved motifs can be identified within the C-proximal protease domain of the P1s of members of the *Potyvirus* genus. The consensus sequence of these motifs, including the enzymatic active site, is: **H**-(x_8)-**D/E**-(x_{28-31})-G-x-**S**-G-(x_{10-21})-I/V-I/V-R-G (residues of the catalytic triad are in bold; Fig. R1). DsMV differs slightly from the rest of potyviruses in having the conserved Glu and His of the P1 protease active site separated by

*[3] This chapter is an adaptation from the article of the same title published in *Journal of General Virology* (2007) 88, 1016-1028. I have maintained the data reported in this article (corresponding to potyviral genomic sequences available in international databases in July 2006). However, the discussion takes into account also data published more recently.

nine residues. The cleavage site between P1 and the next protein in the potyviral polyprotein, HCPro, is also well conserved. It is 22-28 aa downstream of the strictly conserved RG dipeptide, and has as consensus sequence I/V/L/M-x-H/E/Q-F/Y↓S. However, exceptions are found at all positions except the Phe/Tyr located right before the cleavage point (Adams *et al.*, 2005a, and Fig. R1).

Virus species	Motif H-x8-D/E	Motif GxSG	Motif RG	Cleavage site⇩	Other motifs	pI
Potyviruses	* *	* **	**			
BCMV Y	353LPHED-GKYKKKE	26IKPGDSGLLFD	14MIIRGRSNGK----LVNALDEQQ	3SIHHYS	1,2,5	9.3
BCMV R	333LPHES-GKYKRKE	26IRPGDSGLVFD	14MIIRGRLNGR----LVNALDEQQ	3SIHHYS	1,2,5	9.1
WMV	353LPHEE-GKYVHQE	26LTHGDSGLLFD	14FVVRGRENGK----LVSAFEEFR	3DIQHYS	1,2,5	9.1
WVMV	227LPHES-GIYRATE	26ITYGDSGLLFD	14FCVRGRRFGK----LVSAFDTHK	3EIQHYS	1,2,5	9.5
EAPV	345LPHAD-GIYKNRE	26IKRGDSGLVFD	14FVIRGRCNGK----LVNALDFIK	3SVVHYS	1,2,5	9.5
SMV	219LPHEE-GKYIHQE	26ITYGDSGLLFD	14FVVRGRKNGK----LVNALEVVE	3DIQHYS	1,2,5	9.7
BCMNV NL-3K	325LCHES-GRKVHRE	26LCKGDSGLLFD	14FIVRGRQEGQ----LVCATEYLD	3TIEHYT	1,2,5	9.1
BCMNV NL-3D	227LCHES-GRKVHRE	26LCKGDSGLLFD	14FIVRGRQEGQ----LVCATEYLD	3TIEHYT	1,2,5	9.7
CABMV	196LPHEE-GVHVHEE	26FRKGDSGLVYP	14FVVRGRLDGS----LINALDWCS	3HVQHYS	2,5	9.6
TFMV	266LPHMR-GEKLAQE	28IKPGDSGIVYN	13FIVRGKLNGR----LIDARSYME	5SIKEFS	1,5	9.9
ZYMV	220VAHEE-GRMRHTE	26IKPGCSGWVLG	14LVIRGRDDDG----IVNALEPVL	3EVDHYS	1,2,5	9.9
BtMV	221LPHHN-SNYICRE	27IAIGWSGLILP	13VIVRGRLYGR----VEDARTKLP	5RTMHYS	1,2,5	10
PeMoV	230LAHEQ-GRRLRRE	27VTYGWSGAILN	13VVIRGRLYGK----LVDARSKLS	5KIHQYS	1,2,5	10
DsMV	291LPHERRGTRLRVE	27VTHGWSGIVMD	14FVIRGRLGGK----LVSACDNFL	5RIVQYA	1,2,5	10.6
LYSV	268TKHTL-GLKRKVD	28IKKGWSGFVLQ	15FVVRGSSRGK----LVDACARIN	4KLEYFS	1,5	8.4
OYDV	363TLHES-GIINRLD	27IRKGHSGLIIN	16MVVRGRLYGR----VVDSLLKIH	5DIEHYS	1,5	8.9
SYSV	374TEHEK-GRLSRKD	27IQKGSSGLIIR	16MVIRGRKNGS----LVDSLLTLK	5DVDHYS	1,5	8.4
MDMV	150TRHEN-NQFKRVD	28IHKGHSGLTFI	05FIVRGRLRGE----LCNSLDCTK	3EIEHYA	5	9.8
SrMV	150TRHEM-GNYKRKD	28IRKGDSGITYI	05FIIRGKHQGK----IINSIEEVN	3EIDHFS	5	9.7
SCMV	150TRHEN-GLFKRKD	28LNRGSSGLTFM	05FIVRGRMHGE----IVNSLHDSK	3EIEHYA	5	10.3
PeMV	163TLHEE-GKFKRRD	28LRKGDSGLTCF	05FIVRGRCDNE----LVNSLTIEN	3DINHYA	5	9.9
JGMV	152TNHEQ-GRITRRD	28FSKGSSGITFK	06FVIRGRVNGV----LVNALDQYE	4QICHYS	5	9.6
CSV	219TRHET-GLKVGLD	28LTKGSSGYLID	10FVVRGRYNSI----LLPSTQILP	5GMVEYS	1,5	10
LMV	342TAHEE-GHRRRVD	28LSPGSSGYVLN	15FIVRGRVDGE----VIDSQSKVT	5RMVQYS	1,3,4,5	9.1
JYMV	232TKHEE-GFNKARD	28IKPGHSGFVLN	15FIVRGNHEGK----LYDARIKLS	5KIVRFA	1,3,4,5	10
PSbMV	304TKHHK-GIISQKD	26ITHGHSGVVFL	15FVVRGKRNGK----LMESRNKVA	5QIDHFS	1,5	9.9
ChiVMV	205TLHHR-GIYQNVD	28ISIGDSGLCIP	15FTVRGRYGSL----LIDSQAYLP	5RINHYS	1,2,5	9.9
KoMV	235TKHHE-GRRSQRD	27ITYGDSGLAID	14TIIRGECEGK----IFDARSKVT	5RMKQFA	1,2,5	10
YMV	204TKHMD-GARKKID	27LTYGDSGRIIL	14LIVRGEHEGK----IYDARVKVT	5TMRQFS	1,2,5	9.8
PVY	189TAHMM-GLRRRVD	28IRRGDSGVILN	15FIVRGSHEGK----LYDARSRVT	5SMIQFS	1,2,3,4,5	10.4
PepSMV§	205TRHET-GVKRRSI	27IYQRVTAAQFE	15FIVRGEHEGK----LYDARSRVS	5QMTHFS	1,2,3,4,5	10.4
WPMV	194LAHMK-GSRKRVD	28LQRGDSGVVLN	15FIVRGEYEGK----IFDARSKLT	5RMIQYS	1,2,3,4,5	10.3
PVV	194LAHVQ-GKRRRVD	28LRRGDSGVILQ	15FIVRGAYEGK----IFDARSKLT	5RMVQFS	1,2,3,4,5	10.1
PTV	194LAHMR-GIRKRVD	28VRRGDSGMILQ	15FIVRGSFEGK----ILDARSKLT	5RMIQFS	1,2,3,4,5	10.4
PepMoV	192LHHMR-GVDRKRD	28LKRGDSGLILN	15FIVRGKSDGV----VLDARSKLS	5HMEQYS	1,2,3,4,5	10.2
LMoV	215VAHHE-GLRRRRD	28IKAGDSGIVLL	15FVVRGNHEGK----LYEARRKVT	5RMIHYS	1,2,3,5	10.8
TuMV	267VAHLL-GKRAQRD	28VCAGWSGIVVG	15FVIRGEHEGK----LYDARIKVT	5KIVHFS	1,3,4,5	10.3
ScaMV	116VKHLQ-GRRQRRD	28IEAGWSGFLLN	15FVVRGKCADT----LFDARVRMT	5NIRQFS	1,5	10.3
PPV	213VRHLD-GSKPRYD	28VTPGMSGFVVN	15FIVRGKHNSI----LVDSRCKVS	5EIIHYS	1,2,3,4,5	9.9
TEV	211VRHMY-GRRKRVD	27LTFGSSGLVLR	14FIVRGRSDGM----LVDARAKVT	5SMTHYS	1,5	10.2
PRSV	453LRHMN-GIRARQD	28LTFGSSGLIFK	14FVVRGRLGGK----LFDGRSKLA	5KMEQYN	*1*,5	9.3
SPFMV	569LKHME-GLRESVD	28LSKGSSGLVLN	15LVVRGALRGV----LYDARMKLG	5YIIQYS	1,3,4,5	9.2
PVA	204LKHHD-GRMHRRD	27LTHGSSGLVFW	15IIVRGRCNGI----LVDARAKLS	5STHHYS	1,5	10.3
PLDMV	385LRHLR-GELRRKD	28VRRGHSGHIIQ	15IIVRGSMDGR----IIDARSKIT	5NMTHYS	1,5	9.2
DapMV	204VYHLR-GRKPRID	29VKAGCSGFVFR	15FIVRGRHEGK----LYDARLKVT	5TMHHYS	1,*5*	9.9
BYMV	189LNHMV-RKRRRVD	25LRKGHSGLVLQ	14TIVRGVVNEGNIPVLVDARKKLS	5TIREFS	1,5	10
ClYVV	207VNHMV-RKRRSVD	25LREGHSGLVLQ	14TIVRGVIKSHGVPCLVDARQNLN	5RIREFS	1,5	9.9
TVMV	180VAHAK-GHRRRID	27LRKGDSGIVLL	15FIVRGTCDDS----LLEARARFS	5RATHFS	1,*3*,5	10.4
Rymoviruses						
AgMV	158TQHEF-KKLVRTD	28IKPGHSGAVLA	06FIVRGIAETM----LVDAREHFK	4RIQHFS	1,5	9
HoMV	158TQHEM-KRIRRTD	28IKPGHSGAILK	06FIVRGISNNM----LVDARTHVI	4AIEHFS	1,5	9.7
RGMV	170TKHHK-GVLSSRD	27IKPGDSGLIYR	08RVVRGRHGGE----IIDARDYVR	4TIKHYS	1,5	10
Ipomoviruses						
CVYVa	439VRHLN-GCNPEVD	27IVPGTSGLITV	08TIIRGWLDR-----IVDARENLT	5RIRNYT	1,5	8.5

CVYVb	218LNHIF-GLDFDDDMDPEI	23ICPGWSGVVIT	18CVVQGKNRES---GIIENAIIMK	6KIDFYC		5.1
SPMMV	637LRHAT-RDISKSGEDDMY	29VRPGWSGVILH	18CVVQGRNLIT---NKIENALEKK	6QIQFYA	1	5.4
Tritimoviruses						
BStMV	308LRHVL-ESNNVDPSDDLY	24IKPGWSGVVLL	16FVVQGIGPD----GQLKNALKTT	3RIEYYS		6
ONMV	255LKHTL-GYPKREWDPLKD	26VTHGWSGIVLS	16FVVMGRCAH----GRLQNALRPT	3GLRWYS		7.4
WSMV	255LKHTM-GYPKRDWDATKD	26VTLGWSGVLLS	16FIVMGRCAH----GRIQNALKPK	3GLRWYG		7.4

Figure R1. Features conserved in P1 proteins of the family *Potyviridae*. Asterisks indicate the catalytic residues H, D/E and S as well as other residues that are invariable in potyviral and rymoviral P1s. Dashes represent gaps. The most conserved residues are boxed in particular colours according to chemical similarities. Distances to the N-end of the protein or to the previous conserved motif (in numbers of amino acids) are indicated upstream each sequence.

☩ Motifs that can be recognized in each P1 sequence: 1, motif FG (Fig. R2); 2, motif FLxG (Fig. R5); 3, motif ISIxGG (Fig. R4); 4, motif TPS (Fig. R4); 5, motif VELI (Fig. R3). Motifs weakly conserved are in italics.

§ It appears that a frameshift has occurred in the reading of the sequence between just upstream the Asp of the catalytic triad and the beginning of the conserved motif RG.

The divergence in size and sequence of potyviral P1s upstream of the protease domain prevented us from obtaining a confident phylogenetic tree of the complete sequences. Even analysis of the conserved C-terminal protease domain using a fragment that spanned from two residues upstream of the conserved H of the catalytic triad to the cleavage site, did not provide reliable phylogenies (data not shown). The frequent occurrence of recombinations is known to affect phylogenetic analysis (Posada & Crandall, 2002). In our study, many branches of the tree that the computer program provided were not well supported by the bootstrapping data, such that only the previously described BCMV (Ward & Shukla, 1991), SCMV (Shukla *et al.*, 1992), and PVY (Spetz *et al.*, 2003) subgroups could be recognized. As a result, we looked for conserved motifs in the extremely variable N-terminal domains by visually analyzing alignments that were derived from members of known groups, and searched equivalent motifs in P1 sequences from other viruses. This strategy allowed us to identify several motifs, as discussed below (Fig. R1).

A highly conserved motif with the consensus sequence, N-end ($x_{4\text{-}6}$)-n-I-m-F-G-S/T-F-e-C-k-L, was detected in members of the PVY subgroup (residues in upper and lower case letters were found in at least five and three or four species, respectively) (Fig. R2). Careful inspection detected similar motifs with a more relaxed consensus sequence around a conserved Gly in most potyvirus species and in three rymoviruses (Fig. R2). However, we were unable to identify this signature in viruses from the SCMV subgroup. Interestingly, although this motif was primarily located near the N-terminus of the protein, in some viruses it was located more internally.

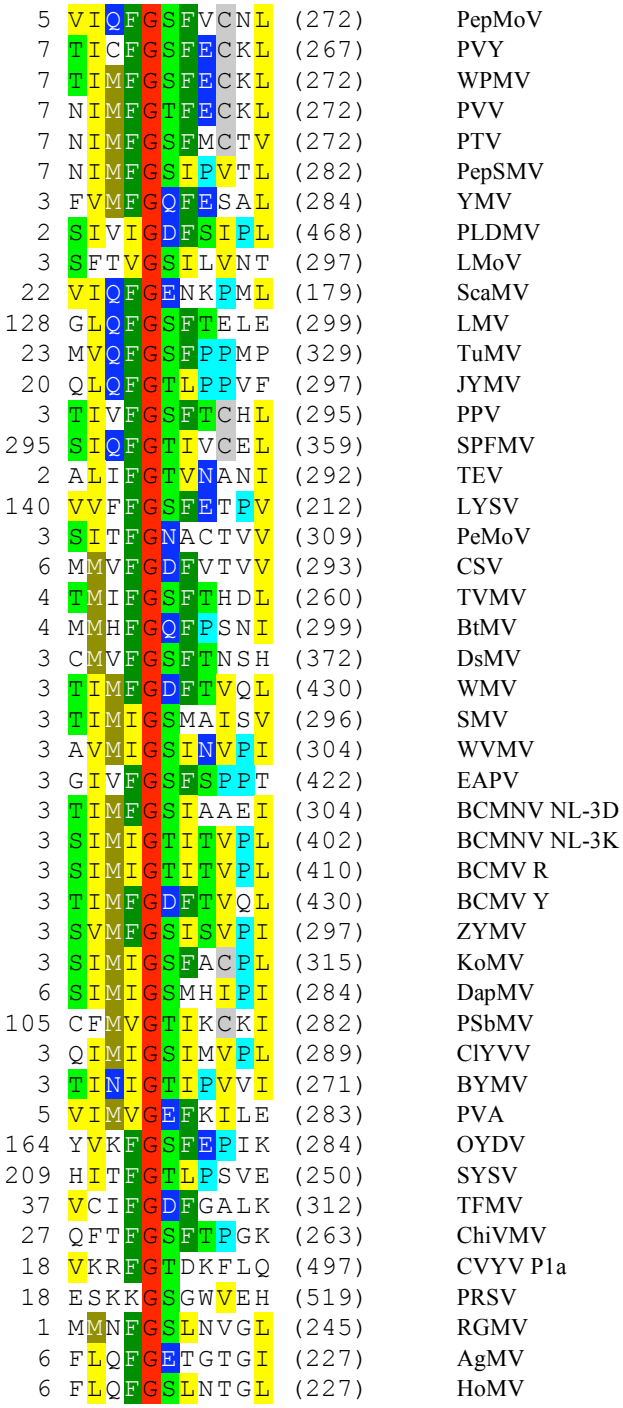


Figure R2. Amino acid sequence alignment of the conserved IxFG motif of potyviral and rymoviral P1 proteins. The position of the first residue is indicated at the left, and the number of amino acids downstream the aligned fragment in each P1 protein is shown in parenthesis at the end of the sequence. The most conserved residues are boxed in particular colours according to chemical similarities.

Another ubiquitous motif was detected 11-21 aa upstream of the catalytic His (Fig. R3). This motif is characterized by a Glu residue preceded by one hydrophobic residue (mainly Val), and followed by another two hydrophobic amino acids, Gly, and between two and five

basic amino acids (Lys or Arg) in the next five positions. However, the consensus sequence of this motif is very relaxed, and none of its residues are conserved in all potyvirus sequences.

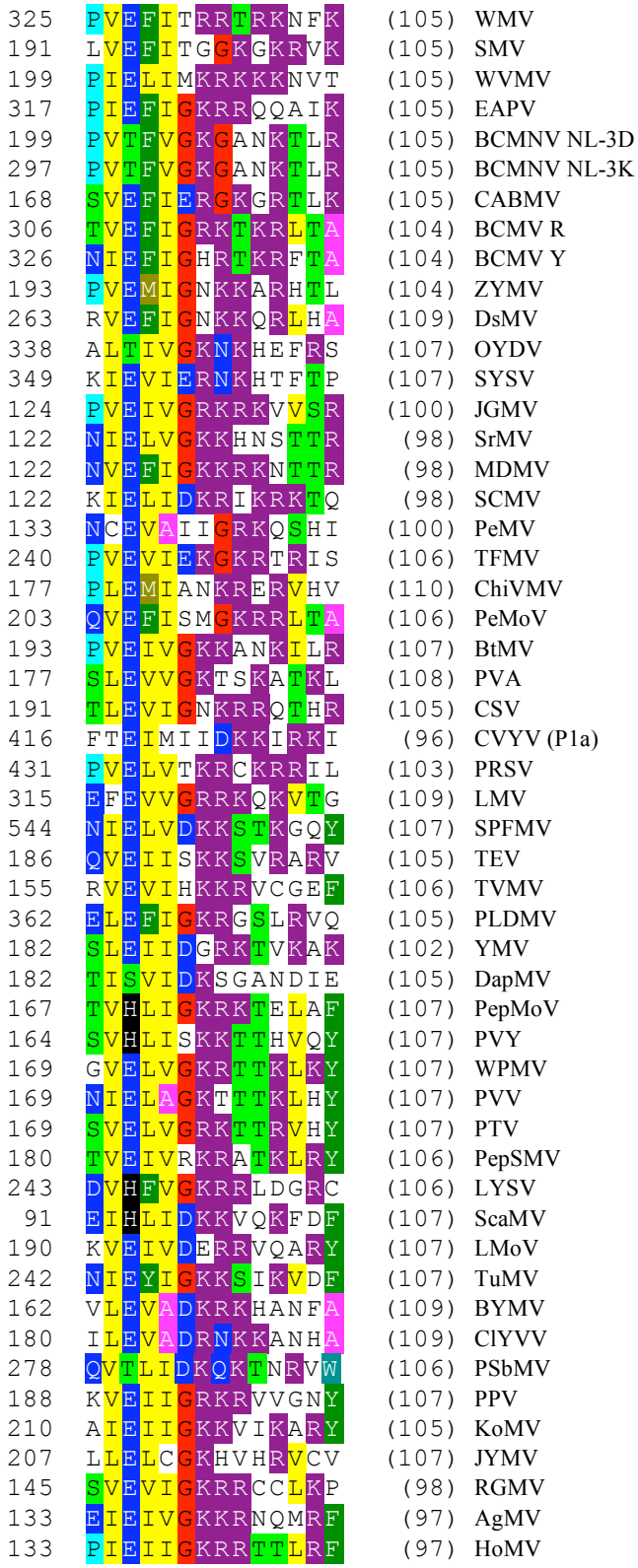


Figure R3. Amino acid sequence alignment of the conserved VELI motif of potyviral and rymoviral P1 proteins. The position of the first residue is indicated at the left, and the number of amino acids downstream the aligned fragment in each P1 protein is shown in parenthesis at the end of the sequence. The most conserved residues are boxed in particular colours according to chemical similarities.

We detected other motifs that were conserved in smaller sets of potyviruses. Two sequential conserved motifs separated by 9 or 10 aa were placed 57-58 aa upstream of the His in the catalytic triad of viral P1s from the PVY subgroup. Consensus sequences of the two motifs were P-s/y-I/v-V/i-S/t-x-I-s/t-I/v-A/g-G-G-x2-p-S and p-l/i-h/n-k/t-T-P-S/r-x-K/r-x-k (residues in upper case letters were found in at least five out of the six subgroup species) (Fig. R4). The two motifs were detected at the same distance from the protease domain in five potyviruses that did not belong to the PVY subgroup: LMV, SPFMV, TuMV, PPV, and JYMV. Although these viruses did not cluster together in the phylogenetic tree of the P1 protease domains, probably because of low resolution outside of the three main potyvirus subgroups (data not shown), all of them were closely linked within the complete phylogenetic trees (see, for instanceAdams *et al.*, 2005b; Petrzik & Franova, 2006). In contrast, these motifs could not be identified in ScaMV, a close relative of TuMV, at the full genome scale. Although the first of these motifs was well conserved in LMoV, and still recognizable in TVMV, the second motif was not visible in these two viruses, suggesting that P1 evolved irregularly (Fig. R4).

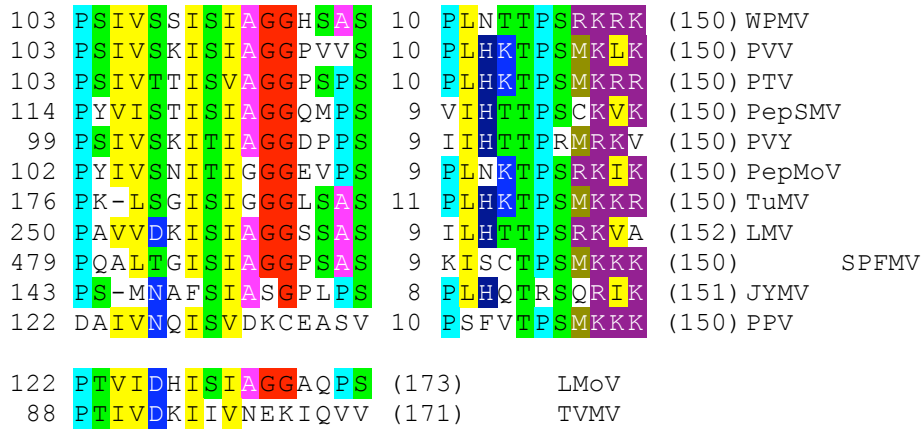


Figure R4. Amino acid sequence alignment of the conserved ISIxGG and TPS motifs of potyviral P1 proteins. The position of the first residue is indicated at the left, and the number of amino acids downstream the aligned fragment in each P1 protein is shown in parenthesis at the end of the sequence. The number of amino acids separating the two motifs is also indicated. The most conserved residues are boxed in particular colours according to chemical similarities. Dashes represent gaps.

A distinctive motif of viral P1s from the BCMV subgroup was found 92-96 aa upstream of the catalytic His (between 94 and 252 aa from the N-terminus of the protein) (Fig. R5). It has the consensus sequence E-E-e-a-F-L-a-G-x-Y-e (residues in upper case letters were found in at least nine of the twelve subgroup species). More degenerated forms of this motif were located at the same distance from the protease domain in viral P1 proteins from the PVY subgroup, as well as in ChiVMV, PeMoV, BtMV, TVMV, LMoV, PPV, and YMV. No

simple phylogenetic relationships justify the presence or absence of this motif. Interestingly, TFMV, the only potyvirus that shares with BCMV subgroup members and their closest relatives, BtMV and PeMoV, the peculiarity of having a Glu instead of an Asp within the P1 catalytic triad (Fig. R1), lack this conserved motif (Fig. R5).

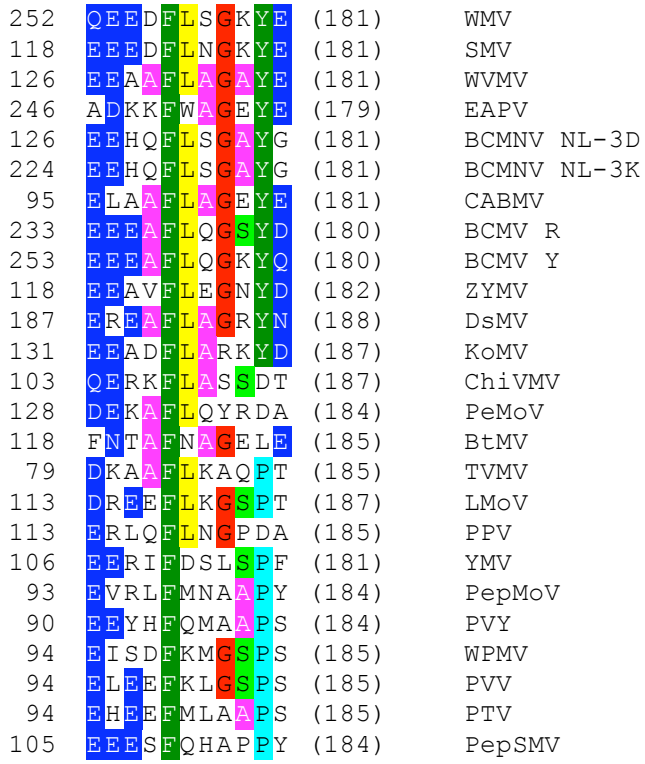


Figure R5 Amino acid sequence alignment of the conserved FLxG motif of potyviral P1 proteins. The position of the first residue is indicated at the left, and the number of amino acids downstream the aligned fragment in each P1 protein is shown in parenthesis at the end of the sequence. The most conserved residues are boxed in specific colours according to chemical similarities.

All these results suggest that the potyviral P1 gene has undergone extensive and uneven evolutionary diversification that has not always paralleled the evolution of the complete genome.

III.1.2 Recombination events in potyviral P1 evolution

To investigate the suspected frequent recombination affecting the P1 region of potyviruses, we decided to select a few examples in which the recombination events could be easily inferred by protein sequence comparison, and confirm those cases using bioinformatics approaches. Published evaluations of the available methods of recombination detection were considered in order to select the most satisfactory one (Kosakovsky Pond *et al.*, 2006; Posada, 2002).

Sequence alignment of the BCMV subgroup viruses suggests that WMV may have resulted from a recombination event in the P1 genes of BCMV and a SMV-related potyvirus (Desbiez & Lecoq, 2004). The presumed crossover region of WMV is shown in Fig. R6A. We performed further sequence alignment analysis and included potyviruses outside the BCMV subgroup. The BCMV-derived region of WMV included sequences that are very similar to sequences from the completely unrelated potyvirus, PLDMV (Fig. R6A). Interestingly, BCMV/PLDMV similarity ended upstream of the BCMV/SMV recombination site of WMV (Fig. R6), suggesting that the BCMV-related parent of WMV was indeed a recombinant virus.

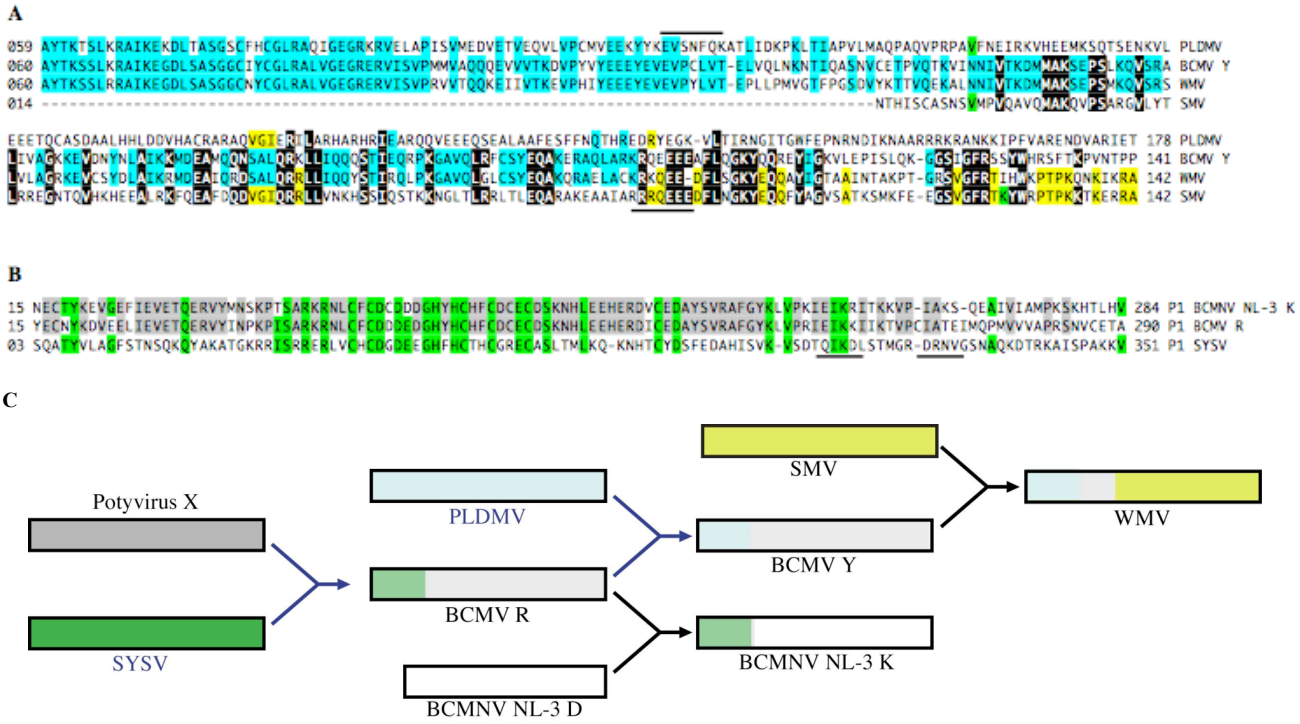


Figure R6. Recombination events at the P1 coding sequence involved in the generation of BCMV-related viruses. A) Partial amino acid alignment around the putative recombination sites of BCMV Y and WMV. Boxed amino acids are identical in BCMV Y and at least one of the other aligned sequences (grey boxes), in SMV and at least one of the other aligned sequences (cross-hatched boxes) or in BCMV-Y and SMV (black boxes). The position of the first residue in each row is indicated on the left, and the number of amino acids downstream of the aligned fragment in each P1 protein is shown in parentheses at the end of the sequence. B) Partial amino acid alignment around the putative recombination sites of BCMNV NL-3 K and BCMV R. Amino acids in black boxes are identical in SYSV and at least one other aligned sequence; residues in grey boxes are identical in BCMNV NL-3 K and either BCMV R or BCMNV NL-3 D, but are not conserved in SYSV. The first residue of the aligned P1 fragments and the number of downstream amino acids (in parentheses) are indicated on the left and right sides of the sequence, respectively. In panels A and B, dashes represent gaps, and stripes indicate the putative recombination sites, which coincide with those detected using GARD analysis of the whole P1 nucleotide sequences (see text for details). C) Evolutionary pathway proposed for BCMV-related viruses. Boxes represent the P1 proteins and the different fillings indicate the viruses that might supply the different regions. Recombination events involving potyviruses outside the BCMV subgroup are shown with dashed arrows.

The BCMV P1 sequence used by Desbiez *et al* (2004) for alignments which detect the recombinant origin of WMV originated from BCMV Y (AJ312438). The P1s from other

BCMV isolates, such as the previously named *Peanut stripe virus* (AY968606, U34972, U05771), also included the PLDMV-related sequence, however this fragment was not present in the P1 of the BCMV isolates BCMV R, RU-1, and the isolate previously identified as *Blackeye cowpea mosaic virus* (AJ312437, AY863025, AY575773). A BLAST search using the N-terminal region of these P1s as a query revealed that they were very closely related to the equivalent region in the NL-3 K isolate from BCMNV (AY864314), but not to other BCMNV isolates (NL-3 D, AY138897, AY282577, U19287). This supports a previous report that found that BCMNV NL-3 K was a natural recombinant derived from BCMNV and BCMV (Larsen *et al.*, 2005). Interestingly, the P1 region that was similar in some BCMV and BCMNV isolates also showed notable similarity to the N-terminal region of SYSV, a potyvirus that does not belong to the BCMV subgroup. The sequence similarity between SYSV and the BCMV R- and BCMNV NL-3 K-like isolates was much lower than the similarity between PLDMV and the WMV and BCMV Y-like isolates (Fig. R6), suggesting that the recombination event involving the SYSV relative occurred much earlier than recombination involving PLDMV. In addition, the observation that the SYSV-related regions of the BCMV R- and BCMNV NL-3 K-like isolates were more similar to each other than to SYSV (Fig. R6B) suggests that these isolates did not derive from independent recombination events involving SYSV, but were evolutionarily sequential. This agrees with the suggestion that BCMNV NL-3 K is derived from recombination between BCMV RU1 and BCMNV NL3-D (Larsen *et al.*, 2005).

For further confirmation of these visually detectable putative recombination events, corresponding GARD analyses (Kosakovsky Pond *et al.*, 2006) were performed. We began by testing the SMV, BCMV-Y, and WMV sequences. The analysis showed a high score (c-AIC score improvement of 58.2) for a single breakpoint that coincided with the previously described recombination event (Desbiez & Lecoq, 2004). A similar analysis performed with PLDMV, BCMV-R, and BCMV-Y sequences located another single recombination site (c-AIC score improvement of 61.1) slightly downstream of the breakpoint predicted from the protein alignment (Fig. R6). Finally, the SYSV, BCMV-R, BCMNV NL-3D, and BCMNV NL-3K sequences were analyzed using GARD tests for multiple recombination, showing two breakpoints corresponding to positions 445 (amino acid residue 102) and 508 (amino acid residue 123) in the BCMNV NL-3K sequence (Fig. R6). The neighbour-joining trees that were derived from automatic analysis of the corresponding fragments between the recombination sites supported the expected relationships (data not shown).

After considering all the recombination events, a potential evolutionary pathway was designed for these potyviruses (Fig. R6C). An early recombination event between the SYSV ancestor and another potyvirus would have produced the BCMV R precursor. Recombination between BCMV R-type isolates and PLDMV and a BCMNV NL-3 D-type isolate would have produced the BCMV Y-type and BCMNV NL-3 K-type isolates, respectively. Finally, WMV would be the result of a third round of recombination between a BCMV Y-type isolate and SMV.

Interestingly, the P1 of another BCMV subgroup potyvirus, EAPV, like the P1 of BCMV-Y-type isolates and WMV, has a PLDMV-related domain. However, in contrast with WMV P1, EAPV P1 does not share close sequence similarity with SMV P1, and it is not evident whether EAPV is derived from a BCMV-Y-type recombinant (by linear evolution or recombination with an unidentified potyvirus), or from an independent recombination event involving PLDMV (data not shown). GARD analysis of EAPV, BCMV-Y, and PLDMV confirmed a putative recombination breakpoint approximately 15 residues upstream of the region where recombination was detected in PLDMV, BCMV-R and BCMV-Y, although with a lower score (Δ c-AIC of 12.2).

Since WMV has a wider host range than SMV, it is suggested that the N-terminal region of P1, the primary feature that distinguishes between these two viruses, is especially relevant for host-virus interaction (Desbiez & Lecoq, 2004). A role for this genomic region in pathogenicity is also supported by the disparate symptomologies caused by the BCMNV NL-3 K- and BCMNV NL-3 D-type isolates. However, in this instance, differences within the P1 N-terminus do not appear to affect virus host range (Larsen *et al.*, 2005). Further support for the importance of P1 in host range definition is provided by the finding that one *Pinellia ternata* potyvirus is closely related to SMV, with the exception of the P1 gene, which resembled the P1 from another BCMV subgroup member, DsMV (Chen *et al.*, 2004b). Sequence alignment analysis suggested that this virus might have derived from a recombination event that occurred at a point close to the P1/HCPro junction (Fig. R7A). The DsMV/SMV recombinant (SMV-P) differs from typical SMV isolates in its ability to infect *Pinellia*, but maintains the ability to infect some soybean cultivars (Chen *et al.*, 2004b). Interestingly, we have observed DsMV-related sequences in the N-terminal region of the P1 gene from the potyvirus KoMV (Nishiguchi *et al.*, 2006) (Fig. R7B). Corresponding GARD analysis of the aligned nucleotide sequences of SMV, SMV-P, DsMV, and KoMV supports evidence for multiple recombination breakpoints, with the P1/HC-Pro site having the highest score (Fig. R7A), followed by the upstream recombination site predicted from the protein

alignment (Fig. R7B). KoMV does not belong to the BCMV subgroup and is most closely related to YMV (Nishiguchi *et al.*, 2006). Both KoMV and DsMV are shown to infect different species of the *Araceae* family (Lesemann & Winter, 2002), which also includes *Pinellia*, the natural host of the DsMV/SMV recombinant. These results further support a role for the N-terminus of P1 in potyvirus host range selection.

A

```
SMV      240 PYICMFAKY-KSINADDITYGDSGLLFDERSSLTTNHTKLPYFVVRGRRN
SMV-P    259 QRLARAVKHSQIIHDNEITYGWSGIVMDQRQQLMHKITRNSSFVIRGRLD
DsMV     314 QRLAGAVRYVKEIHDSEVTHGWSGIVMDQRQQLIHKISRSSIFVIRGRLG

SMV          GKLVNALEVV--ENMEDIQHYSQNPEAQFFRGWKKVFDKMPPHVENHECT 2730
SMV-P        GRLVDACEILPRNTLHQIVQFSKTPEAQFFIGWKKMFDKMLPHVENHECT 2747
DsMV         GKLVSACDNFLWDNALRIVQYAKTPEAQFFQGWREVFAGMGPQQGDHGCS 2778
                                   P1   HCPro
```

B

```
DsMV 146 SLFKKSHAKLVRGRNQSWRLSTPTMEIAEERQAALDRERQEREAFLAGRYNPEDVVGG
KoMV  90 SLFKKSHAKLVRGRKQGWRLSTPSLEVAQQRQAKIDELMQEEADFLARKYDPQDVIGG

DsMV     YVDIRDRTKRGEQISFKGPFWKRTPKTPRVVK---KQPRFVVANAFKVERALLRSLQH 126
KoMV     HVLVRDQTKRGEQVSFKGPFWHRTYKTKHTRTNTLSSPRMDETSLLGLVRGVFKIAKA 123
```

C

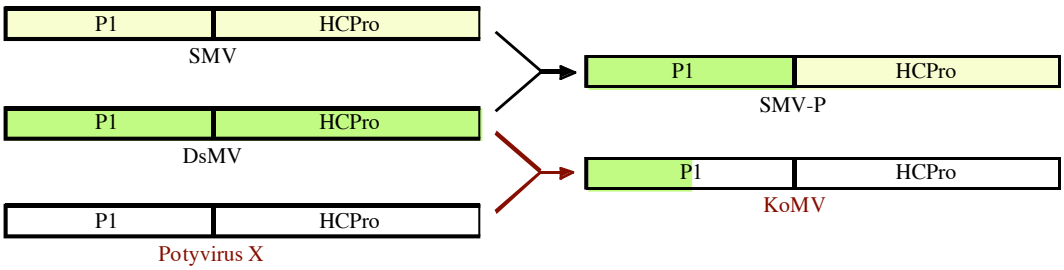


Figure R7. Putative recombination events involved in the generation of the *Pinella* isolates of SMV (SMV-P, AJ507388) and KoMV. A) Partial amino acid alignment around the suggested recombination site of SMV-P. Boxed amino acids are identical in SMV-P and the SMV severe strain (SMV, AJ312439) (cross-hatched boxes), in SMV-P and DsMV (grey boxes), and in the three viruses (black boxes). The first residue of each row is indicated on the left, and the number of amino acids downstream of the aligned fragment of each potyviral polyprotein is shown in parentheses at the end of the sequence. The border between P1 and HCPro is also indicated. B) Partial amino acid alignment around the putative recombination site of KoMV. Amino acids in grey boxes are identical between KoMV and DsMV. The position of the first residue of each row is indicated on the left, and the number of amino acids downstream of the aligned fragment of each P1 protein is shown in parentheses at the end of the sequence. In panels A and B, dashes represent gaps and stripes indicate the putative recombination sites, which coincide with those detected using GARD analysis of the whole P1 nucleotide sequences (see text for details). C) Schematic representation of the RNA recombination events. Boxes represent the P1 proteins and the different fillings indicate the viruses that supply the different regions. Recombination events involving potyviruses outside the BCMV subgroup are indicated with dashed arrows.

III.1.3 Atypical P1 proteins in tritimoviruses

As mentioned above, our attempts to derive phylogenetic relationships from the P1 of potyviruses were unsuccessful. However, when members of other genera from the *Potyviridae* family were incorporated into the analysis of the conserved C-terminal protease region of P1, some interesting features were observed. First, rymoviral P1s did not cluster apart from potyviral P1s, which supports the assessment that *Rymovirus* is the closest genus to *Potyvirus* (Adams *et al.*, 2005b) (data not shown). In contrast, tritimoviral P1s constituted an independent branch in the tree, which is consistent with previous phylogenetic reports of the *Potyviridae* family (Adams *et al.*, 2005b). In addition, some features clearly distinguished tritimovirus P1s from rymovirus and potyvirus P1s. The distance between the Asp or Glu, and His residues in the catalytic triad of potyvirus and rymovirus P1s is 8 aa in 49 of the 50 species analyzed, and 9 aa in DsMV (Fig. R1). In contrast, these residues were separated by only 7 aa in the P1s from tritimoviruses (Fig. R1). Moreover, there is a conserved motif between the Ser residue of the catalytic triad and the autocleavage site, which contains an invariable dipeptide Arg-Gly in all potyviral and rymoviral P1s. However, the Arg residue was replaced by Gln or Met in tritimoviral P1s (Fig. R1).

The most conspicuous difference between the two types of P1s was their isoelectric point (pI). In spite of having extreme sequence divergence, a universal feature of potyvirus and rymovirus P1s is their high pI, which is greater than 10 in 20 viral species, between 9 and 10 in 27 species, and between 8.4 and 8.9 in the remaining 3 species. In contrast, the pI of BStMV was 6.0 and the pI of both WSMV and ONMV was 7.4 (Fig. R1). This difference probably not only reflects a large phylogenetic distance, but also some functional divergence.

III.1.4 P1 duplication in ipomoviruses

As mentioned above, potyviruses P1 proteins show a huge size divergence, ranging from the 211 aa of ScaMV to the 664 aa of SPFMV. However, the size of the ipomovirus CVYV P1 reported by Jansen *et al*. (2005) was 843 aa, which is notably higher. These authors identified a P1-like protease domain near the carboxyl end of the protein, with a catalytic triad formed by His 746, Asp 754, and Ser 789, and a putative cleavage site between Tyr 843 and Cys 844. However, another P1-like protease domain was recognized with a catalytic triad formed by His 442, Asp 451, and Ser 484, and a presumed scissile bond between Tyr 525 and Thr 526 (Fig. R1). Cleavage at this site would produce two mature proteins, P1a and P1b, that were 525 and 318 aa, respectively. When the two putative P1 protease domains were included in the phylogenetic analysis, the P1a domain clustered with the potyviral and rymoviral P1s

and the P1b domain was more related to the tritimoviral P1s (not shown). The assignment of CVYV P1a and P1b to each P1 type was strongly supported by the following facts: i) the His and Asp residues of the catalytic triad were separated by 8 and 7 residues in P1a and P1b, respectively (Fig. R1), ii) the Arg-Gly dipeptide was present in the conserved domain downstream of the catalytic Ser in P1a, while Gln-Gly was the dipeptide present at the equivalent position in P1b (Fig. R1), and iii) P1a was a basic protein of pI 8.5, while P1b had a pI of 5.1 (Fig. R1). Evidences indicating that the internal protease domain is functional and cleavage takes place to yield P1a and P1b were obtained by different approaches (see chapters III.2 and III.3).

The P1 protein of SPMMV, the type member of the *Ipomovirus* genus, consists of 743 aa. Sequence analysis revealed a single protease domain at the C-terminus of the protein. This domain clustered with the tritimovirus P1s and the CVYV P1b in phylogenetic analysis. Moreover, the SPMMV P1 had hallmarks of being a tritimovirus-like P1 (Fig. R1): i) the first two residues of the catalytic triad were separated by eight amino acids, ii) the Arg that precedes the invariable Gly of the conserved motif located downstream of the Ser of the catalytic triad was absent, and iii) the protein had a low pI (5.4).

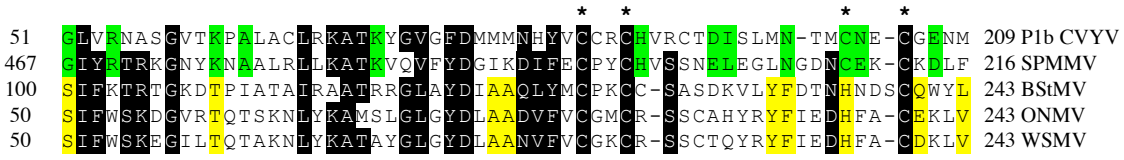


Figure R8. Partial amino acid alignment of ipomoviral and tritimoviral P1s. Boxed amino acids are identical or chemically similar between the two ipomoviral sequences (cross-hatched boxes), between the three tritimoviral sequences (grey boxes), and between at least four of the aligned sequences (black boxes). Dashes represent gaps. The position of the first residue of the aligned P1 fragments and the number of downstream amino acids are indicated on the left and right sides of the sequence, respectively. P1b of CVYV is considered to begin at aa 526. Cys and His residues that likely compose a Zn finger structure are shown with asterisks.

Sequence conservation upstream the protease domain of tritimo-like P1s was rather poor. However, there was a conserved Cys-rich domain that resembled a Zn finger (Fig. R8). The third of four Cys residues that compose the putative Zn finger was replaced by His in the P1 proteins of the tritimoviruses analyzed.

Zn finger-like sequences are not a general feature of potyviral P1s. However, Cys and His residues that may form part of Zn finger structures were detected in several potyviruses: PLDMV/BCMV-Y/EAPV/WMV, SYSV/BCMV-R/BCMNV NL-3 K, OYDV/PSbMV, LYSV/LMV, and PRSV/SPFMV (Figs. R6A, R9, and R10). The functional relevance of these putative Zn fingers remains unknown.

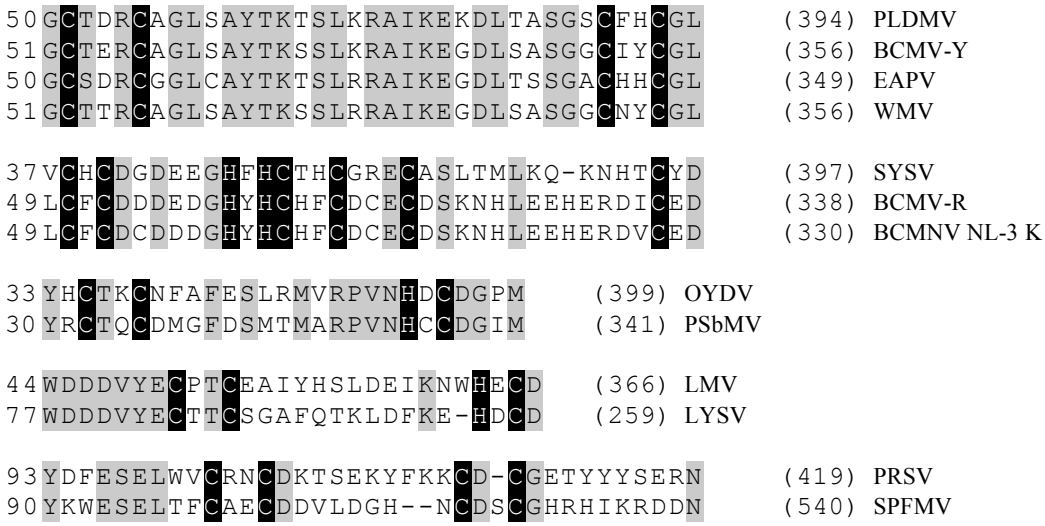


Figure R9. Amino acid sequence alignment of cysteine rich domains of potyviral P1 proteins. The position of the first residue is indicated at the left, and the number of amino acids downstream the aligned fragment in each P1 protein is shown in parenthesis at the end of the sequence. Cys and His residues conserved in each set of compared sequences are boxed in black, other conserved residues are boxed in grey. Dashes represent gaps.

The P1 protein of some potyviruses enhances the activity of the RNA silencing suppressor HCPro, but does not have its own silencing suppression activity (Kasschau & Carrington, 1998; Rajamäki *et al.*, 2005). The ipomovirus CVYV lacks an HCPro gene, and its tritimo-like P1b protein appears to compensate for this defect, as it has been shown to have RNA silencing suppression activity similar to that observed in the potyviral HCPro (see section III.2).

III.1.5 Evidence for intergenera recombination between ipomovirus and potyvirus P1s

Sequence alignment of the P1 regions of the ipomoviruses SPMMV and CVYV only showed noticeable similarity at their last 300 aa. Thus, we performed a BLAST analysis of the remaining sequences. The P1a protease domain of CVYV showed clear homology to the P1 protease domains of potyviruses and rymoviruses. Interestingly, sequence similarity to CVYV P1a extended upstream to the N-terminus of the P1 protein from a single potyvirus species, PRSV, with an E-value of $4.8e^{-15}$ in the BLAST search (Fig. R10B and data not shown). Moreover, the N-terminus of the ipomovirus SPMMV P1 was very closely related to the potyvirus SPFMV P1 (E-value of $4.3e^{-39}$). Strong similarity between the SPMMV and SPFMV P1s ended approximately 183 aa upstream of the His in the SPFMV catalytic triad and 58 aa upstream of the SPMMV region that is similar to CVYV (Fig. R10); no significant similarity was detected for these 58 aa in any other proteins. Some similarity was also detected between the N-terminal regions of PRSV and SPFMV (E-value of 0.082), suggesting that these sequences may share a common ancestor. Interestingly, the four sequences shared

four conserved cysteines (Fig. R10) that resembled the Zn finger-like motif at the N terminal region of the tritimo-like P1s (Fig. R8). This would support a model in which the N-terminus of the P1 regions of the potyviruses PRSV and SPFMV and the ipomoviruses CVYV and SPMMV, and the N-terminal region of the tritimovirus-like P1s, derived from a preceding P1 duplication. In this scenario, SPMMV would derive from an ancient ipomovirus that harboured two copies of the P1 gene, by deletion of the protease domain of the first copy and the first amino acids of the second copy, and the SPFMV P1 would have resulted from a recombination event between the ipomovirus SPMMV and an unknown potyvirus (Fig. R10). Given the high similarity between the homologous SPMMV and SPFMV sequences, this putative recombination event should have occurred recently. Similarly, PRSV P1 would have resulted from a recombination event between the ipomovirus CVYV, which retains both P1 copies, and an unknown potyvirus (Fig. R10). In this second case, the recombination event could have occurred much earlier, such that the recombination site would not be easily recognized. Attempts to apply automated tools of recombination detection to these sequences were unsuccessful because of the intrinsic difficulty of aligning sequences with so much divergence (data not shown). However, when a GARD analysis was applied to the PRSV, SPMMV, and SPFMV P1 nucleotide sequences that were arranged according to the amino acid alignment shown in Fig. R10, a single breakpoint was detected at position 1276 (P1 aa 379) in the SPMMV sequence (c-AIC score improvement of 76.6). The high score obtained for this recombination site not only confirmed the presumed breakpoint between SPMMV and SPFMV, but also justified the reliability of the alignment.

The sequence shared by the potyvirus SPFMV and the ipomovirus SPMMV strongly suggests that the N-terminal region of their poty-like P1 is important for fitness within their common sweet potato host. In this respect, it is important to remark that SPFMV and SPMMV are able to coinfect sweet potato (Mukasa *et al.*, 2006), which can facilitate recombination events that result in better adapted viruses. Evidence for a relationship between sequence homology at the N-terminal region of P1 and common host adaptation are less compelling for PRSV and CVYV. However, while *Carica papaya* is the nominal host of PRSV, this virus can also infect cucurbits, the only host of CVYV, and previous studies suggest that the papaya-infecting variants of PRSV may have been derived from cucurbit-infecting ancestors (Bateson *et al.*, 2002).

A.

```
100 WVCRNCD---KTSEKYFKKC-DCGETYYYSERNLMRTMNDLMYQFDMTPSEINSVDLEYLANAVDYAEQL PRSV
100 FEC-ECG---NSNTSWKRVCEDCEQVYAYSEMNLLQELSKIARGLSCYINELKNFTIHDIADEVSYAEKS CVYV
 86 HFCPDCDVIVDSEEGWF--CEDCGSQFNKRDDNVLDNKNDVARALGGWNEYEDATWALFEAARADMLEVA SPMMV
 97 TFCAECDDVLDGHN-----CDSCGHRHIKRDDNIADNMNAIARALGGYDAYYASNWAVYETAKYELDQVA SPFMV

    VKRSQVPEPVELAMMEPIVASGEGILMVSEPEVMPVTTKVEEAWTIQIGEIPVPLVVIKETPVISGVEGT PRSV
    RRMTTVE-------------SGFKT--VGE---ISIRIEVVEA---VISEKPVESVTVKAVVAEKPVEAS CVYV
    PTVGQLE-------------KEIRA--IEK--SAGKKLTAYEE---EMLEELEL----AYKLDVAKMNEEKQ SPMMV
    PTAGMLY-------------KQAKE--AEK--LLGKRPTRREI---QEVEDL--WAEYEEAAAREAAEEAS SPFMV

    LNS---------------------------------------------------------------------- PRSV
    DNSRVAMEEKKPQTKQVWKMV-------------------------------------------------- CVYV
    EEVLEETNFSISNDEFPALNGPQDEEVNVVIEETTEESAIEVAKEAEKSVEFEIIHEKTDEPISDAVNAR SPMMV
    EASNGHATSEVANKNAYLSDGEDDEAFPPLVVTVEKVVPATTIIESTPEVGKTIEVQTPLEPVPEVLAAT SPFMV

    ------------------------------------------------------------TGFSLEADIT PRSV
    ------------------------------------------------------------TGKPKPTVIP CVYV
    MVATPVVATSVTKSGTVIDGKELVEKPKTTMWVTKPKTTAAIPATSSKSAVWVAKPKPASAIFIAEPVVK SPMMV
    TFVEATIDGKDAPTGSIQFGTIVCELEPTKA--SEAEIAKEPTTGFFFGTIPAIVPLPTIPLLKLESTIV SPFMV

    KLVEKEILQEEVKEAV-HLALEVGNEIAE-KKPELKLIPY-WSASLELHKRIRKHKEHAKIAAIQVQKER PRSV
    ECKPEAPTKKEIQTCL-DLVVQIGDFIINTRTREFKMVDE-ENECTEKVEETEV-KEP-------VVENP CVYV
    PAVRACNDVMNIGAMVCPIMVSANAQVEDATKEEEPVIKYNITFGSFNYEVSTKGERIQAAVQLDEIIEG SPMMV
    EPIATPTVVVTSSEIV-KVPIATPTEVEKASKAPLPKHLYPWTAKTQTPGKVHHKMVRKWVQKTQ-QAAA SPFMV

    EKDQKVFSALELRLNLKSRRRNQAVVCDKRGTLKW------ETQRGHKKSKLMQQASDFVVTQIHCDFGC PRSV
    NKDTVVLNDFFKNFQFNEERRSK-IYTDKHGNVRYGK----RPMKKRKRGKKVELVSDKIITKIETPEEV CVYV
    PDIEPILICQTGSSHKSETKKAAKGLFVQD----------------------------------------- SPMMV
    EKEKLVWKKLDEQLATRNEIRKDLKVKWRWGLYRLVKKTRKDNQRQRRQRRMEKEQQLLMAMPPQALTGI SPFMV

    KTQYSEPHIPGIKQSTSKKICKPRKHSRIVGNSKINYIMKNLCDTIIE-------RGIPVELVTKRCKRR PRSV
    QPVYSFEDLPSIKKAYSKK--KPRE----IIKTRTEYSVRSLVKEIGK-------TCSFTEIMIIDKKIR CVYV
    ---------------------------------------------------------------------- SPMMV
    SIAGGPSASLEMTPTPNGKISCTPSMKKKKTLKSPRLTQEKIHELTQAVLKIACRKRMNIELVDKKSTKG SPFMV
                      *       *                                   *
    ILQKEGRSYV---QLRHMNGIRARQDVSSSPDMELIFTQFCKFLVGHKPLKSKNLTFGSSGLIFKPKFAD PRSV
    KIKKRGSRYF--VDVRHLNGCNPEVDLDHSSFSDEILDWLMASLAVKSTKLS-EIVPGTSGLI------- CVYV
    ---------------------------------------------------------------------- SPMMV
    QYKKFQGANYLFLHLKHMEGLRESVDLRIHTTTQNLVLQAAKVGAWKRTVKTTMLSKGSSGLVLNPDKLL SPFMV

    NVGRY-FGDYFVVRGRLGGKLFDGPSKLARSVYAKMDQY                                  PRSV
    TVGRNEFGKFTIIRGWLD-RIVDAPENLTKSQLRRIRNYTIHGLHAFKKRYQTNAIDRECTTQIQIKDNV 287 CVYV
    ----------------------------------------------------------------KFSVIGNK 296 SPMMV
    GPRGHAPHGMLVVRGALRGVLYDARMKLGRSVLPYIIQY                                  SPFMV
```

B.

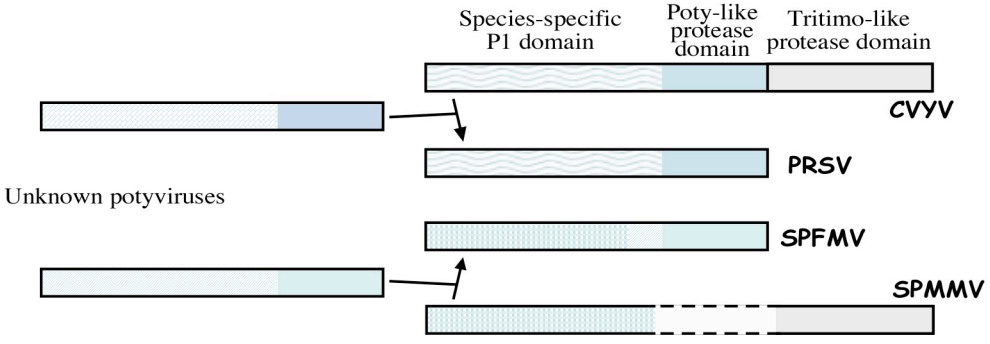


Figure R10. Partial amino acid alignment of the P1 proteins of the ipomoviruses CVYV (P1a+P1b) and SPMMV and the potyviruses PRSV and SPFMV. Boxed amino acids are identical or chemically similar between PRSV and CVYV (grey boxes), between SPMMV and SPFMV (cross-hatched boxes), and between the four aligned sequences (black boxes). Dashes represent gaps. The position of the first residue of each row is indicated

on the left, and the number of amino acids downstream of the aligned fragment of each P1 protein is shown in parentheses at the end of the sequence. The catalytic triad is shown with asterisks and the cleavage sites that separate P1 from HCPro in PRSV and SPFMV, and P1a and P1b in CVYV are indicated with arrows. The region of the putative recombination site involving SPFMV and SPMMV, which coincides with that detected using GARD analysis on whole P1 nucleotide sequences, is underlined (see text for details). B) Schematic representation of the RNA recombination events that may have produced the existing viral sequences, and the putative parental lineages that are involved.

III.2 RNA silencing suppression by a second copy of the P1 serine protease of CVYV, a member of the family *Potyviridae* that lacks the cysteine protease HCPro[*4]

The full-length genome sequence of CVYV isolate confirmed its assignment to the genus *Ipomovirus*. However, CVYV differed from the type member of the genus, SPMMV, and from most monopartite members of the family *Potyviridae,* in that it lacks a coding sequence for a putative HCPro. CVYV showed an outstandingly large P1 protein, with a C-terminal serine protease domain similar to those of the P1s of members of the genus *Potyvirus* (Janssen *et al.*, 2005). The putative cleavage site separating CVYV P1 and P3 was also similar to the P1-HCPro junction of potyviruses. The lack of HCPro opened up debate about how CVYV could secure the multiple functions of this protein in viral replication and transmission.

Here, we show that the originally described long P1 of the ipomovirus CVYV is really formed by two homologous proteins, P1a and P1b, and that P1b is able to suppress RNA silencing in a similar way to HCPro from potyviruses, suggesting that P1b is replacing HCPro at least in this function.

III.2.1 The N-terminal region of the polyprotein of CVYV includes two P1-like serine proteases

In the report of the full-length genome sequence of the CVYV, Jansen *et al*. (2005) noticed the presence of a P1-like serine protease domain characterized by a H746-D754-S789 catalytic triad, with the serine residue in a GXSG context, as well as a predicted cleavage site IDFY:C (aa 840-844) upstream of the P3 protein, which is in agreement with the consensus sequence for potyviral P1 cleavage sites. Further sequence analysis revealed the presence of an additional P1-like serine protease domain (H442-D451-S484 catalytic triad) and a putative internal cleavage site IRNY:T (aa 522-526), which would split the P1 region in two proteins, P1a and P1b, showing 24% amino acid identity. In order to verify the protease activity of the internal domain, we made two constructs to express by infiltration of *A. tumefaciens* either the complete P1 sequence from CVYV (p35S-$P1_{CVYV}$-CTAP) or its P1b fragment (p35S-$P1b_{CVYV}$-CTAP), fused to a TAP tag (Fig. R11A). For simplicity, in this report, we will refer to each *A. tumefaciens* strain by the plasmid it carries. Western blot analysis specific for the

[*4] This chapter is an adaptation from the article of the same title published in *Journal of Virology* (2006) 80, 10055-10063.

TAP tag showed the accumulation of a protein of ~60 kDa, the size of P1b-TAP, in the *N. benthamiana* leaves infiltrated with p35S-P1b$_{CVYV}$-TAP, at 3 and 6 dpi (Fig. R11B). A minority protein with the expected mobility of unprocessed P1-TAP (120 kDa) was detected at 3 dpi in leaves infiltrated with p35S-P1$_{CVYV}$-TAP (Fig. R11B). However, the major protein at 3 dpi and the only one detected at 6 dpi in these leaves had the same electrophoretic mobility as P1b-TAP expressed from p35S-P1b$_{CVYV}$-TAP, strongly suggesting that P1-TAP was being processed at the predicted internal cleavage site (Fig. R11B).

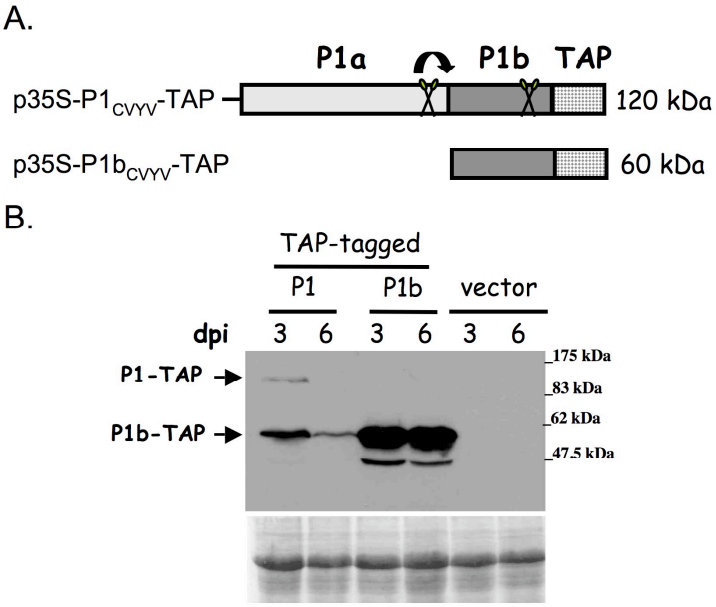


Figure R11. Internal serine protease domain of the CVYV P1 region is functional. (A) Schematic representation of the C-terminal TAP-tagged constructs. The scissors represent serine protease domains, although processing at the end of the second one was not expected because the last residue of P1b was not included in the construct. (B) Western blot analysis of extracts of leaf patches infiltrated with *Agrobacterium* sp. carrying empty pBin19 (vector), p35S-P1$_{CVYV}$-TAP (P1) or p35S-P1b$_{CVYV}$-TAP (P1b), collected at 3 or 6 dpi. The sizes (in kilodaltons) of prestained molecular weight markers (New England Biolabs) run in the same gel are indicated beside the panel. A band of about 45 kDa is present in samples from patches infiltrated with p35S-P1bCVYV-TAP *Agrobacterium sp.* at 3 or 6 dpi. This minor band is recognized by the TAP antibodies, and may represent a partial degradation product. The blot stained with Ponceau red is shown at the bottom as a loading control.

III.2.2 CVYV P1b suppresses both sense RNA- and dsRNA-triggered RNA silencing

The lack of a sequence coding for the typical potyviral silencing suppressor HCPro in the CVYV genome (Janssen *et al.*, 2005) raised the possibility that the exceptionally large P1 sequence of this virus might contribute to counteract the antiviral defense mediated by RNA silencing. To assess this possibility, we constructed *Agrobacterium* plasmids expressing PPV P1-HCPro, and P1a, P1b or the complete P1 from CVYV (Fig. R12), which were co-agroinfiltrated with p35S:GFP (Fig. R13).

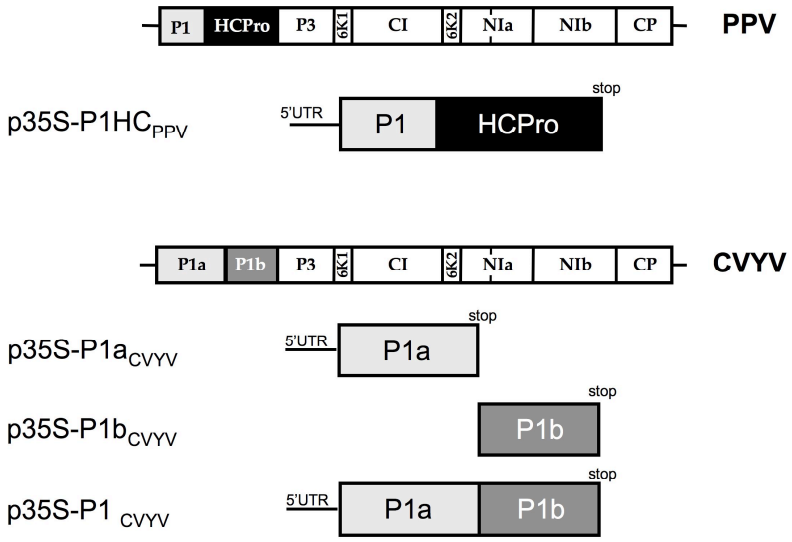


Figure R12. Schematic representation of the PPV-derived and CVYV-derived constructs used in the RNA silencing assays. Genome maps of the viruses are also shown. Stops codons introduced during cloning are indicated.

The green fluorescence at 6 dpi remained as strong in patches coagroinfiltrated with p35S:GFP plus p35S-P1b$_{CVYV}$ as in those expressing p35S:GFP plus p35S-P1HC$_{PPV}$ (Fig. R13A), suggesting that P1b from CVYV could suppress silencing as efficiently as PPV P1-HCPro. A similar GFP fluorescence decline was observed at 6 dpi in leaves infiltrated with p35S:GFP plus either empty pBin19 or p35S-P1a$_{CVYV}$, indicating that P1a does not display silencing suppression activity (Fig. R13A). Very weak fluorescence was observed at 6 dpi in patches expressing GFP and the full-length CVYV P1 (Fig. R13A). This could be due to a low translation efficiency of this protein, since very low P1b accumulation was observed in leaves agroinfiltrated with p35S-P1$_{CVYV}$-TAP, compared with those agroinfiltrated with p35S-P1b$_{CVYV}$-TAP (Fig. R11B), whereas the TAP-tagged products of these two plasmids had no silencing suppression activity (data not shown).

As shown by Northern blot analysis, GFP mRNA accumulation at 3 dpi was similar in leaves infiltrated with p35S:GFP plus pBin19, p35S-P1b$_{CVYV}$, or p35S-P1HC$_{PPV}$, and both CVYV P1b and PPV P1-HCPro were able to prevent, with a similar efficiency, the drop in GFP mRNA levels detected in leaves infiltrated with p35S:GFP plus the empty control plasmid at 6 dpi (Fig. R13B), confirming the silencing suppression activity of CVYV P1b.

Accumulation of siRNAs is a universal feature associated with RNA silencing. As expected, high levels of GFP siRNAs of ~21-24 nt were detected in leaves infiltrated with p35S:GFP plus pBin19 at 6 dpi, when GFP mRNA decline was taking place (Fig. R13B). However, silencing suppression by either PPV P1-HCPro or CVYV P1b did not give rise to an apparent reduction in the accumulation of siRNAs at 6 dpi (Fig. R13B).

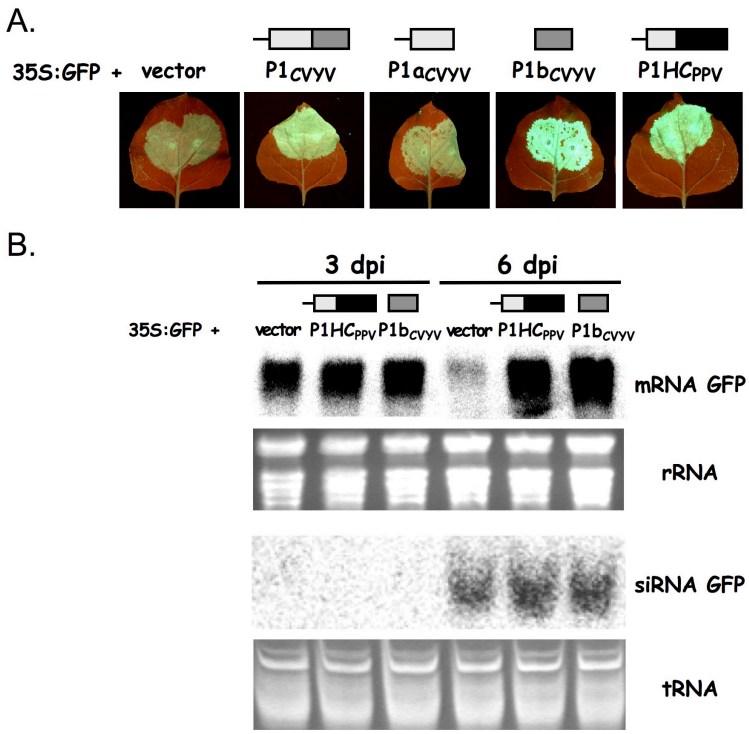


Figure R13. Suppression by CVYV P1b of RNA silencing triggered by GFP mRNA. *N. benthamiana* plants were coinfiltrated with *Agrobacterium* sp. mixtures carrying p35S:GFP and empty pBin19 (vector), p35S-P1HC$_{PPV}$ (P1HC$_{PPV}$), p35S-P1$_{CVYV}$ (P1$_{CVYV}$), p35S-P1a$_{CVYV}$ (P1a$_{CVYV}$) or p35S-P1b$_{CVYV}$ (P1b$_{CVYV}$). (A) GFP fluorescence pictures taken under a UV lamp at 6 dpi. (B) Northern blot analysis of GFP mRNA and siRNA extracted from patches infiltrated with the *Agrobacterium* sp. mixtures indicated above each lane, collected at 3 or 6 dpi. rRNA and tRNA stained with BrEt were used as loading controls for the blots of mRNA and siRNA, respectively.

To induce RNA silencing, the sense GFP RNA must first be converted to dsRNA. In order to assess whether CVYV P1b was targeting this first step or interfering with silencing downstream dsRNA production, we carried out a dsRNA-triggered silencing assay. Leaves were agroinfiltrated with p35S:GFP (GFP sense RNA), p35S:GF-IR (IR generating GFP dsRNA) and pBin19, p35S-P1b$_{CVYV}$, or p35S-P1HC$_{PPV}$. IRs are strong inducers of RNA silencing and, thus, all infiltrated leaves showed only weak green fluorescence under UV light at 3 dpi (data not shown), which dropped to undetectable levels at 6 dpi in patches infiltrated with p35S:GFP+p35S:GF-IR+pBin19, but was maintained, and even increased, in patches expressing PPV P1-HCPro or CVYV P1b (Fig. R14A).

Northern blot analysis corroborated the fluorescence observations. PPV P1-HCPro and, more efficiently, CVYV P1b enhanced GFP mRNA accumulation, both at 3 dpi and 6 dpi (Fig. R14B), supporting the postulation that CVYV P1b, as the potyviral HCPro, was able to interfere with dsRNA-triggered RNA silencing. GFP-specific siRNA accumulation was detected at 3 dpi, indicating that RNA silencing was already induced at this time, but the much higher siRNAs levels at 6 dpi indicated a progressive strengthening of the silencing

response (Fig. R14B). Suppression of dsRNA-triggered silencing by PPV P1-HCPro or CVYV P1b, like that of the sense RNA-triggered one commented above, did not abolish siRNA accumulation, which appeared to be even greater in leaves expressing the PPV P1-HCPro silencing suppressor (Fig. R14B).

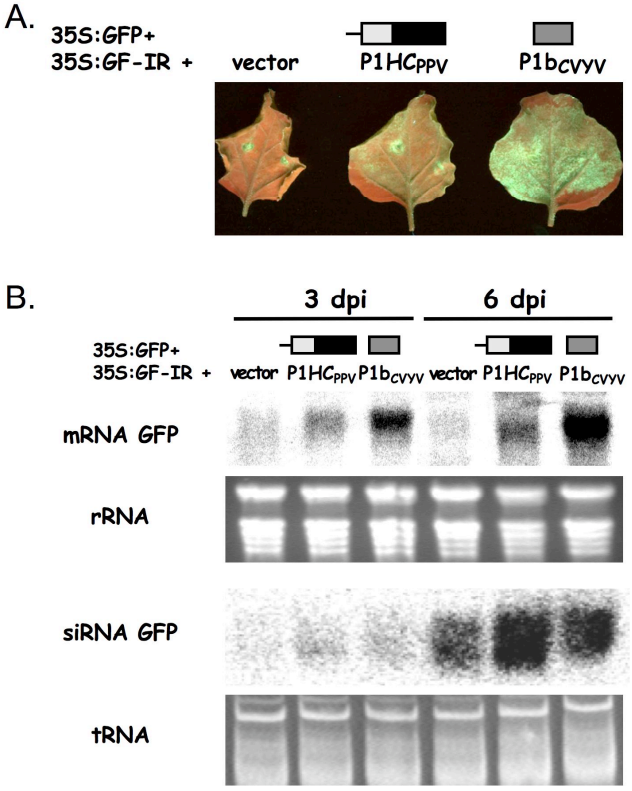


Figure R14. Suppression by CVYV P1b of RNA silencing triggered by GFP dsRNA. *N. benthamiana* plants were coinfiltrated with *Agrobacterium* sp. mixtures carrying p35S:GFP, p35S:GF-IR and empty pBin19 (vector), p35S-P1HC$_{PPV}$ (P1HC$_{PPV}$) or p35S-P1b$_{CVYV}$ (P1b$_{CVYV}$). (A) GFP fluorescence pictures taken under a UV lamp at 6 dpi. (B) Northern blot analysis of GFP mRNA and siRNA extracted from patches infiltrated with the *Agrobacterium* sp. mixtures indicated above each lane, collected at 3 or 6 dpi. rRNA and tRNA stained with BrEt were used as loading controls for the blots of mRNA and siRNA, respectively.

III.2.3 CVYV P1b suppresses local transgene silencing but does not prevent cell-to-cell or long-distance spread of RNA silencing in GFP-transformed N. benthamiana line 16c

In order to verify whether CVYV P1b could suppress not only the RNA silencing induced by transient expression of sense RNA or dsRNA, but also that involving transgene RNA, and to assess the ability of this protein to prevent silencing spread, we agroinfiltrated *N. benthamiana* line 16c, which actively expresses its GFP transgene, with p35S:GFP and plasmids expressing CVYV P1b or PPV P1-HCPro (Fig. R15). Enhanced green fluorescence was observed in the infiltrated patches at 2 to 3 dpi regardless of the expression of silencing suppressors (data not shown), but later declined until it was hardly detectable at 7 dpi in leaves coinfiltrated with p35S:GFP plus the empty vector (data not shown). In contrast, the

green fluorescence remained strong in patches coinfiltrated with p35S:GFP plus either p35S-P1b$_{CVYV}$ or p35S-P1HC$_{PPV}$, for more than 13 dpi (Fig. R15A).

Northern blot analysis showed similar GFP mRNA levels at 3 dpi in the patches infiltrated with any of the bacteria combinations. At 7 dpi, the steady-state level of GFP mRNA was much higher in patches infiltrated with p35S:GFP plus either p35S-P1b$_{CVYV}$, or p35S-P1HC$_{PPV}$ than in those infiltrated with p35S:GFP plus the control plasmid (Fig. R15B). In contrast with the results obtained in wild type *N. benthamiana*, suppression of RNA silencing by PPV P1-HCPro and CVYV P1b in *N. benthamiana* line 16c caused a drastic reduction in GFP-specific siRNA levels, which was especially marked for CVYV P1b (Fig. R15B).

It has been reported that RNA silencing can spread cell-to-cell from agroinfiltrated patches. In GFP-transgenic lines, this spread provokes shutting down of GFP expression in the neighboring cells, which is manifest by a narrow red ring around the infiltrated spot (Voinnet & Baulcombe, 1997). These red rings were observed around all patches infiltrated with p35S:GFP plus pBin19 by 6-7 dpi (not shown). Expression of PPV P1-HCPro or CVYV P1b appeared to cause a delay in short distance spread of silencing, and patches with bright green fluorescence surrounded by red borders were observed in only some leaves infiltrated with p35S:GFP plus p35S-P1HC$_{PPV}$ at 7 dpi (not shown). However, the patches of all leaves expressing PPV P1-HCPro showed red rings at 13 dpi (Fig. R15B). At this time, only 1 out of 50 patches of leaves infiltrated with p35S:GFP plus p35S-P1b$_{CVYV}$ showed the red line (Fig. R15B), but this proportion increased to ~50% at later times (24 out of 48 patches at 23 dpi). Thus, neither PPV P1-HCPro nor CVYV P1b was able to completely block short distance spread of RNA silencing.

Monitoring of upper noninfiltrated leaves of the agroinfiltrated plants at 13 dpi, showed that they were starting to lose GFP fluorescence around major veins, regardless of the fact that the infiltrated patches were silenced or were expressing high levels of GFP owing to the local silencing suppression activity of PPV P1-HCPro or CVYV P1b (Fig. R15C). This demonstrated that PPV P1-HCPro and CVYV P1b also fail to block the long-distance spread of the systemic silencing signal.

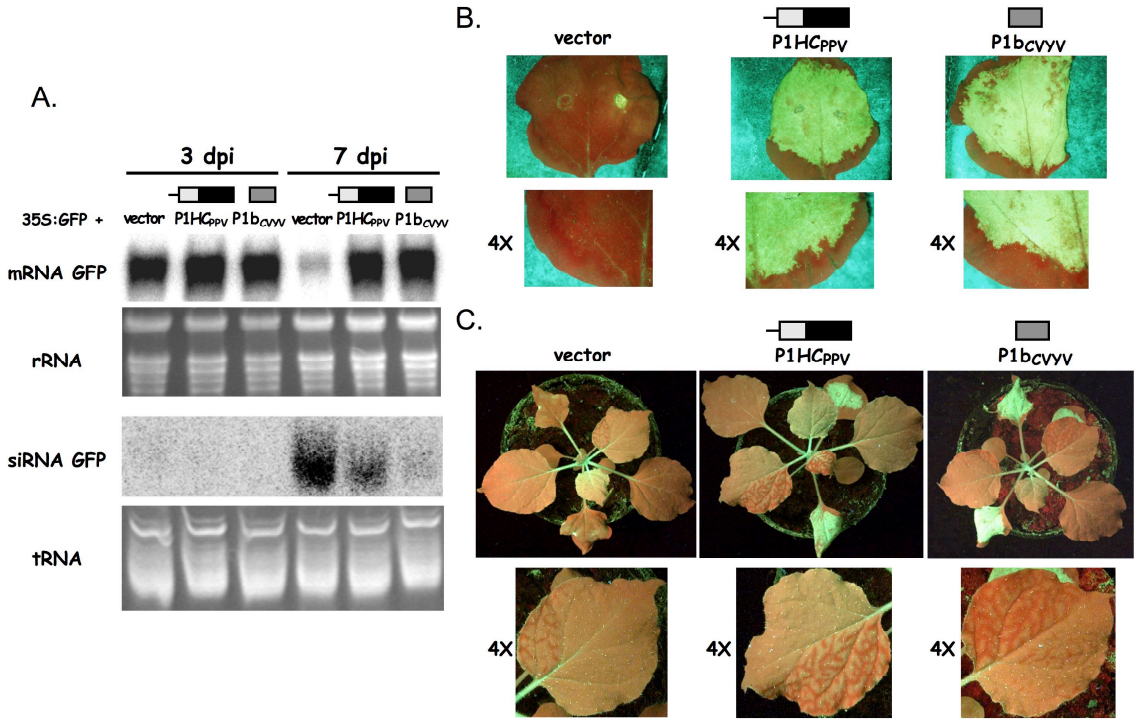


Figure R15. Effect of CVYV P1b on systemic GFP silencing. *N. benthamiana* line 16c plants were coinfiltrated with *Agrobacterium* sp. mixtures carrying p35S:GFP and empty pBin19 (vector), p35S-P1HC$_{PPV}$ (P1HC$_{PPV}$) or p35S-P1b$_{CVYV}$ (P1b$_{CVYV}$). (A) GFP fluorescence pictures taken under a fluorescence microscope at 13 dpi. (B) Northern blot analysis of GFP mRNA and siRNA extracted from patches infiltrated with the *Agrobacterium* sp. mixtures indicated above each lane, collected at 3 or 7 dpi. rRNA and tRNA stained with BrEt were used as loading controls for the blots of mRNA and siRNA, respectively. (C) GFP fluorescence pictures taken under a UV lamp at 13 dpi. Pictures in the lower rows of panels A and C were taken at 4 times higher amplification that those of the upper rows.

III.3 Protease activity, self interaction and siRNA binding of the silencing suppressor P1b from CVYV[*5]

A large number of viral RNA silencing suppressors have been identified. They are extremely diverse in sequence, structure and mechanism of action. Given the key role played by siRNAs and long dsRNAs in the silencing pathways, it has been proposed that sequestering of these molecules could be a general strategy used by viral silencing suppressors (Silhavy & Burgyán, 2004).

In the previous chapters it has been shown that the ipomovirus CVYV lacks the typical potyviral silencing suppressor HCPro but this deficiency is compensated by duplication of the P1 gene, whose downstream copy codes for a silencing suppressor that act in a similar way to HCPro. In the present chapter some biochemical features of CVYV P1b are analyzed. The results demonstrate that P1b is a protease that cleaves at its C-terminus, but that its proteolytic activity is not essential for silencing suppression. It is also shown that CVYV interacts with itself and with siRNAs, and these abilities appear to have functional relevance.

III.3.1 P1b is a serine protease

Amino acid alignment of some P1b-like proteins from ipomoviruses and tritimoviruses, showed a well-conserved C-terminal region, which corresponds to the serine protease domain, with the catalytic triad formed, in the case of CVYV P1b, by H_{221}, D_{229} and S_{264} (Fig. R16). In addition, this analysis also revealed the presence of two conserved motifs located upstream of the protease domain: a putative zinc finger and a LxKA conserved motif (Fig. R16).

In order to verify the predicted protease activity of CVYV P1b, reporter constructs that coded for fusion products consisting of P1b, the first P3 residue and a C-terminal TAP tag (p35S-P1b-CTAP) (Fig. R17A) were expressed *in planta* by *A. tumefaciens* infiltration. Western blot analysis specific for the TAP tag showed accumulation of a protein of ~22 kDa, the size of TAP, in *N. benthamiana* leaves infiltrated with wild type p35S-P1b-CTAP, suggesting that P1b is self cleaving at its C-end in P1b-CTAP (Fig. R17B, lane 5). In order to verify whether the serine protease domain identified *in silico* was involved in generation of the TAP-related 22K product, we expressed mutated versions of P1b-CTAP in which either of two of the residues of the predicted catalytic triad, H_{221} and S_{264}, were replaced by alanine.

[*5] This chapter is an adaptation from the article of the same title published *in Journal of Virology* (2008) 82, 974-986.

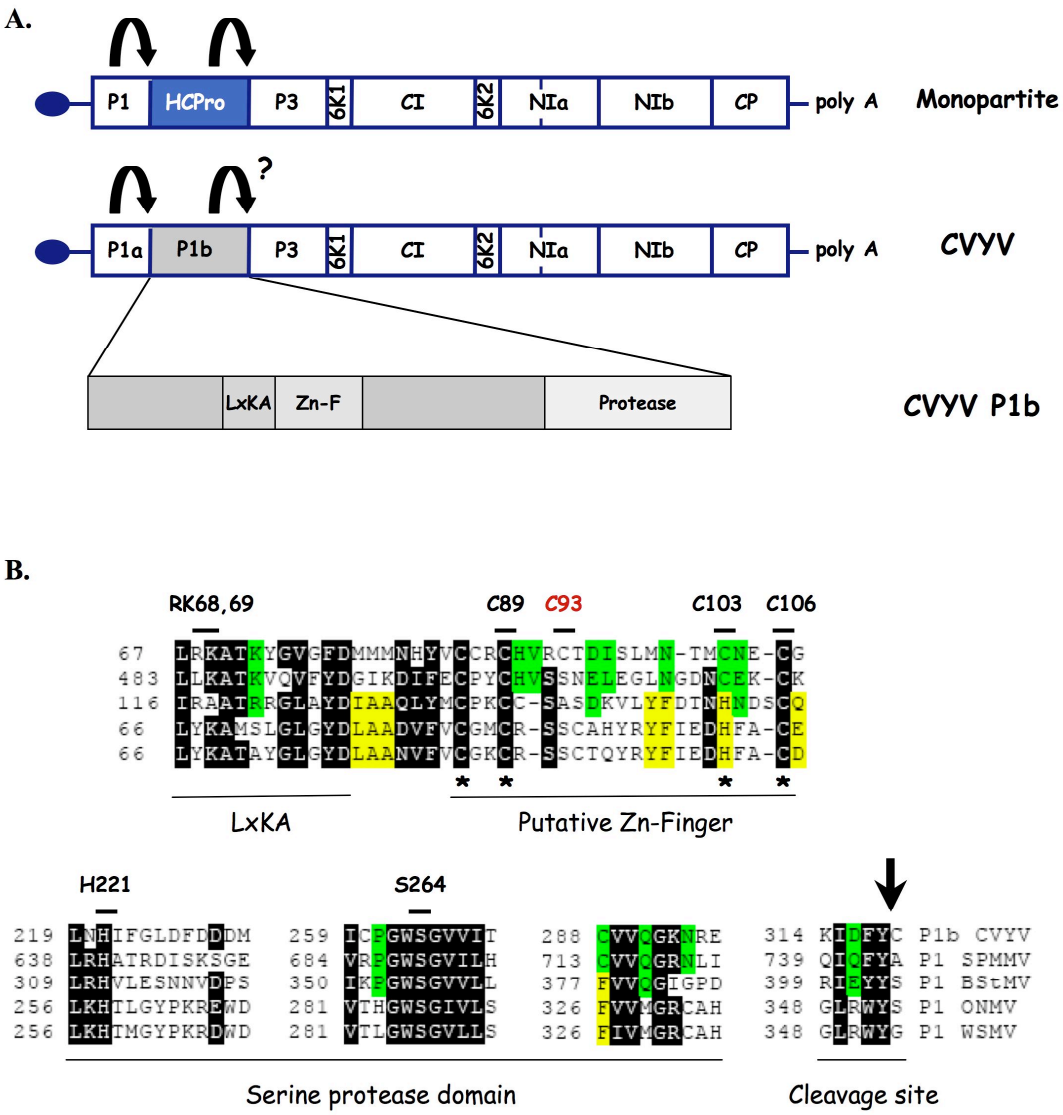


Figure R16. Conserved domains present in P1b-like proteins. (A) Genome maps of monopartite viruses from the family *Potyviridae*. The arrows represent protease activities (the question mark indicates that this proteolytic activity had not been experimentally demonstrated prior to this work). The schematic representation of CVYV P1b shows the location of conserved domains. (B) Partial amino acid alignment of P1b of the ipomovirus CVYV (considered to start at aa 526 of the polyprotein) and P1s of the ipomovirus SPMMV and the tritimoviruses BStMV, ONMV and WSMV. Boxed amino acids are identical or chemically similar between the two ipomoviral sequences (green boxes), between the three tritimoviral sequences (yellow boxes) and between at least four of the aligned sequences (black boxes). Dashes represent gaps. The conserved domains are indicated below the sequence alignment. The position of the first residue of each aligned segment is indicated on the right side of the sequence. Cys and His residues predicted to form a zinc finger domain are marked with asterisks. The arrow indicates the predicted autocatalytic cleavage site. The residues that were mutated in this work, and their position in the P1b sequence, are indicated above the alignment.

The 22K protein was not detected in the TAP-specific Western blot analysis of leaves infiltrated with p35S-P1b-CTAP H221A or S264A, but a protein of ~58 kDa, the size expected for non-cleaved P1b-CTAP, was shown to accumulate in these samples (Fig. R17B, lanes 3 and 4). The most likely interpretation of this result is that P1b indeed cleaves itself in P1b-CTAP and the H221A and S264A mutations abolish the P1b proteolytic activity. To investigate the possible relevance of other P1b regions for protease activity, mutations

affecting the LxKA motif and the putative zinc finger, RK68,69AA and C89A, respectively, were introduced in p35S-P1b-CTAP. Neither of these mutations affected accumulation of the 22K protein (Fig. R17B, lanes 1 and 2), suggesting that the mutated domains were not involved in P1b proteolysis.

This finding and the previous demonstration of the proteinase activity of CVYV P1a (see section III.2.1) strongly suggest that free P1b could be produced in CVYV infection by proteolytic processing of the viral polyprotein. To verify this prediction, anti-P1b serum was produced and used in Western blot analysis to detect P1b-related polypeptides in infected cucumber, the natural host of CVYV. In agreement with the agroinfiltration results, the anti-P1b serum revealed the accumulation of a protein of ~36 kDa, the size expected for free P1b, in CVYV-infected cucumber leaves, which was absent in healthy cucumber leaves (Fig. R17C, lanes 1 and 2). This protein had the same electrophoretic mobility than P1b expressed in *N. benthamiana* leaves by agroinfiltration (Fig. R17C, lanes 3 and 4).

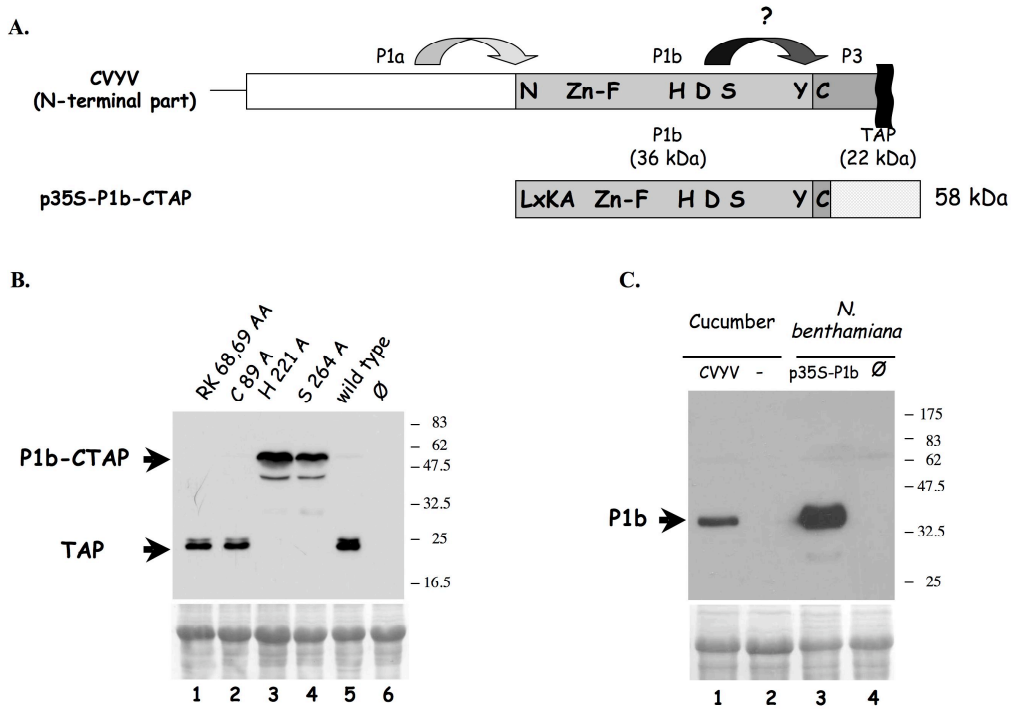


Figure R17. CVYV P1b cleaves at its C-terminus and accumulates in CVYV-infected cucumber plants. (A) Schematic representation of the N-terminus of the CVYV polyprotein and the C-terminal TAP-tagged P1b reporter. Letters inside the boxes represent either conserved amino acids or conserved domains (LxKA: positively charged conserved motif; Zn-F: putative zinc finger; H D S: H221, D229, S264 predicted to constitute the protease catalytic triad; YC: amino acids at the predicted P1b-P3 junction). (B) Western blot analysis with PAP complex of extracts of leaf patches of *N. benthamiana* infiltrated with agrobacteria carrying empty pBin19 (lane 6), p35S-P1b-CTAP (lane 5) or derivatives of this plasmid with the indicated mutations (lanes 1-4), collected at 3 dpi. (C) Western blot analysis with anti-P1b serum of extracts of cucumber leaves systemically infected with CVYV (lane 1) or equivalent leaves of mock-inoculated cucumber plants (lane 2), and leaf patches of *N. benthamiana* infiltrated with agrobacteria carrying p35S-P1b (lane 3) or empty pBin19 (lane 4). The position of prestained molecular mass markers (New England Biolabs) (in kilodaltons), run in the same gels, is indicated to the right of the panels. The blots stained with Ponceau red are shown at the bottom as a loading control.

III.3.2 The putative zinc finger and the LxKA motif, but not the protease activity, are essential for the RNA silencing suppression activity of CVYV P1b

A mutational approach was followed to determine protein domains involved in the RNA silencing suppression activity of CVYV P1b. Mutations were introduced into p35S-NTAP-P1b, which encodes an N-terminal TAP tagged P1b. Unlike C-TAP tagging, which disturbs the silencing suppression activity of P1b, N-TAP tagging while facilitating protein detection and purification, has no appreciable effect on this activity (data not shown). The effect of the mutations was assessed in a dsRNA-triggered silencing assay (Fig. R18). In *N. benthamiana* leaves agroinfiltrated with p35S:GFP (expressing GFP mRNA) and p35S:GF-IR (expressing an inverted repeat which generates GFP dsRNA), GF-IR directed a fast and strong silencing against GFP mRNA and, as a consequence, very weak green fluorescence was detected in infiltrated patches at 7 dpi (Fig. R18A). Consistent with this fact, Northern blot and Western blot analyses showed very low accumulation levels of GFP mRNA and protein, respectively, in these agroinfiltrated leaves (Fig. R18B). Coagroinfiltration with wild type p35S-NTAP-P1b prevented the induction of silencing, and strong fluorescence and high accumulation levels of GFP and GFP mRNA were detected at 7 dpi in leaves agroinfiltrated with the three plasmids (Fig. R18). Patches infiltrated with p35S:GFP, p35S:GF-IR and either the C93A (affecting a non conserved cysteine) or S264A (affecting the protease active center) mutant versions of p35S-NTAP-P1b also showed high GFP expression levels, indicating that these mutations did not affect the silencing suppression activity of P1b (Fig. R18). In contrast, mutations affecting the LxKA conserved motif (RK68,69AA) or the putative zinc finger (C89A, C103A and C106A) abolished silencing suppression activity, and leaves infiltrated with p35S:GFP, p35S:GF-IR and p35S-NTAP-P1b with any of these mutations, expressed GFP at very low levels similar to those of leaves infiltrated with p35S:GFP, p35S:GF-IR and the empty vector pBin19 (Fig. R18). dsRNA-triggered silencing of GFP expression was associated with accumulation of specific siRNAs, however, in agreement with previous results (see section III.2.2), silencing suppression by wild type P1b or P1b C93A and S264A mutants caused only a slight decrease in GFP siRNA levels (Fig. R18). We observed some differences in the amount of siRNAs accumulated in the presence of the different P1b mutants. However, these observations were not exactly reproduced in repetitions of the experiment, suggesting that other unknown factors might affect the siRNA levels resulting in a certain degree of fluctuations in the analysis (data not shown).

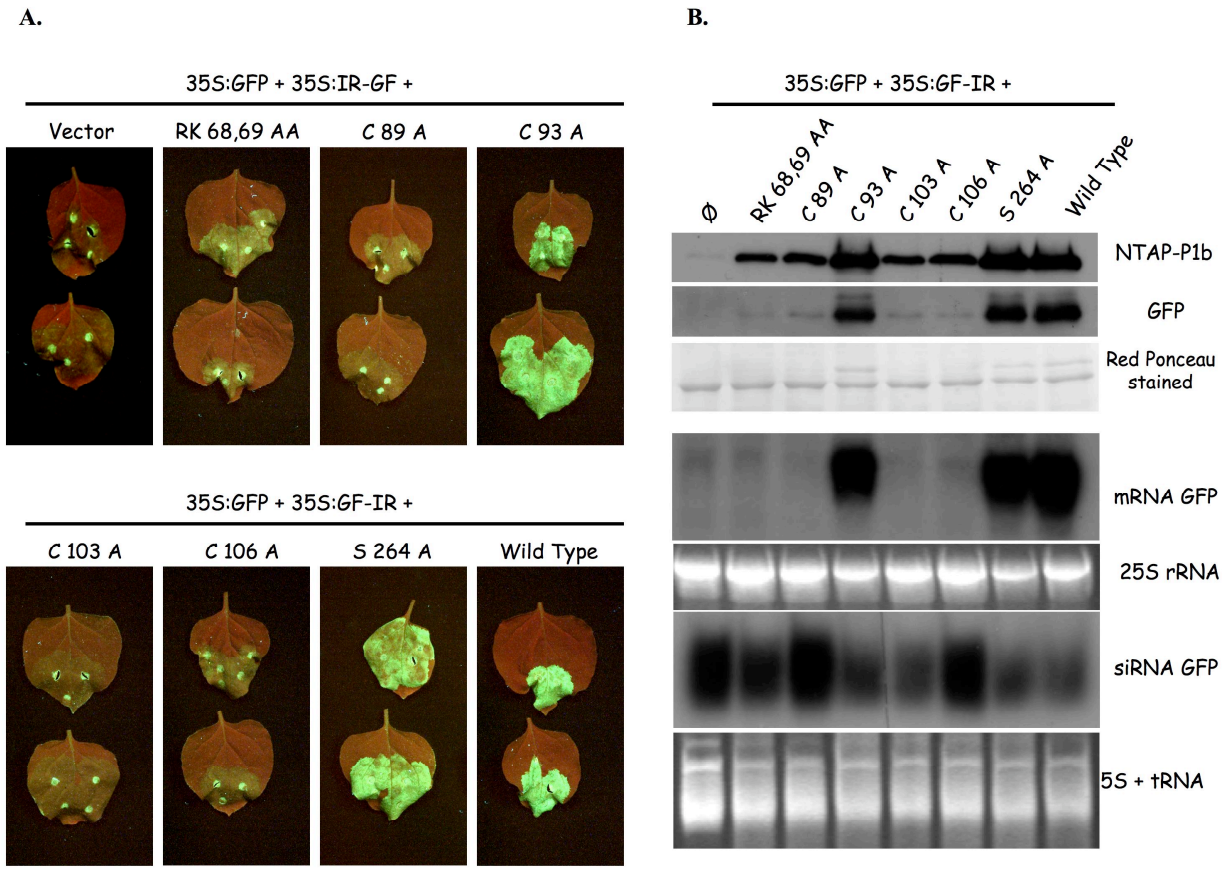


Figure R18. Effects of mutations in CVYV P1b conserved domains on silencing suppression activity. *N. benthamiana* plants were coinfiltrated with agrobacteria carrying p35S:GFP and p35S:GF-IR plus empty pBin19 (Vector), p35S-NTAP-P1b (wild type) or derivatives of this plasmid with the indicated mutations. (A) GFP fluorescence pictures taken under a UV lamp at 7 dpi. (B) Western blot analysis with PAP complex and anti-GFP antibodies (upper panels), and Northern blot analysis of GFP mRNA and siRNA (bottom panels), of infiltrated leaves harvested at 7 dpi. The protein blot stained with Ponceau red and the RNA agarose (25S rRNA) and polyacrylamide (5S + tRNA) gels stained with BrEt were shown as loading controls.

RNA silencing also affected expression of the inactive P1b mutants, and TAP-specific Western blot analysis revealed a lower accumulation of these proteins than those of wild type P1b or of the functional mutants C93A and S264A (Fig. R18). However, at 2 dpi, although the weak sense RNA-triggered silencing affecting p35S-NTAP-P1b expression was still not very effective and all mutant P1b proteins accumulated at similar levels as wild type P1b, dsRNA-triggered silencing already disturbed GFP expression in the absence of P1b or in the presence of P1b with the mutations RK68,69AA, C89A, C103A or C106A (Fig. R19), further supporting the specific effect of these mutations in RNA silencing suppression activity.

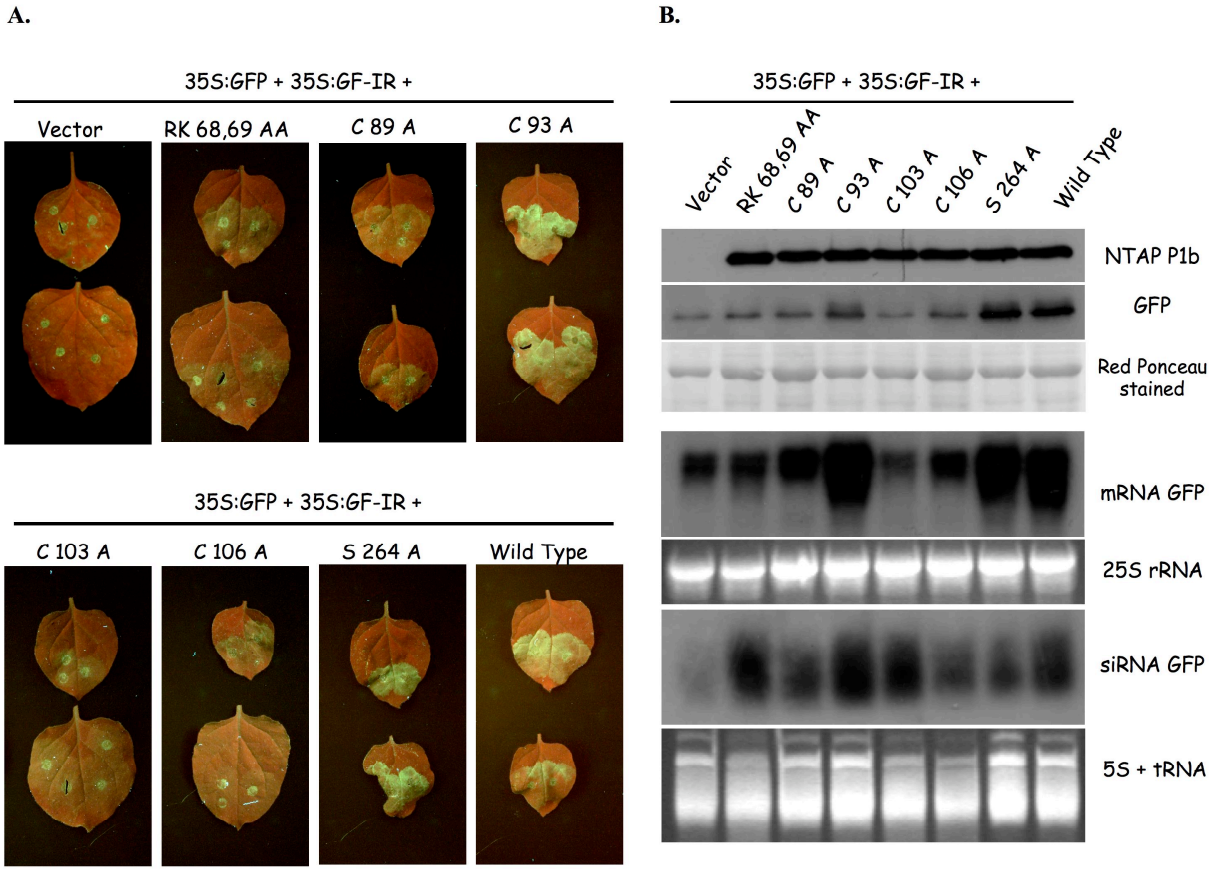


Figure R19. Effects of mutations in CVYV P1b conserved domains on silencing suppression activity. *N. benthamiana* plants were coinfiltrated with agrobacteria carrying p35S:GFP and p35S:GF-IR plus empty pBin19 (Vector), p35S-NTAP-P1b (wild type) or derivatives of this plasmid with the indicated mutations. (A) GFP fluorescence pictures taken under a UV lamp at 2 dpi. (B) Western blot analysis with PAP complex and anti-GFP antibodies (upper panels), and Northern blot analysis of GFP mRNA and siRNA (bottom panels), of infiltrated leaves harvested at 2 dpi. The protein blot stained with Ponceau red and the RNA agarose (25S rRNA) and polyacrylamide (5S + tRNA) gels stained with BrEt were shown as loading controls.

III.3.3 P1b self-interacts in vivo

Previous structural studies of different RNA silencing suppressors revealed self-interactions driving to oligomeric conformations (Chao *et al.*, 2005; Lingel *et al.*, 2005; Plisson *et al.*, 2003; Ruiz-Ferrer *et al.*, 2005; Vargason *et al.*, 2003; Ye & Patel, 2005). In order to assess whether P1b can also interact with itself, we made use of the technique of Bimolecular Fluorescence Complementation (BiFC). BiFC reveals *in vivo* interactions between two proteins by reconstitution of a fluorescing complex from two defective fragments of a fluorescent protein, each one fused to one of the binding proteins (Hu & Kerppola, 2003) (Fig. R20).

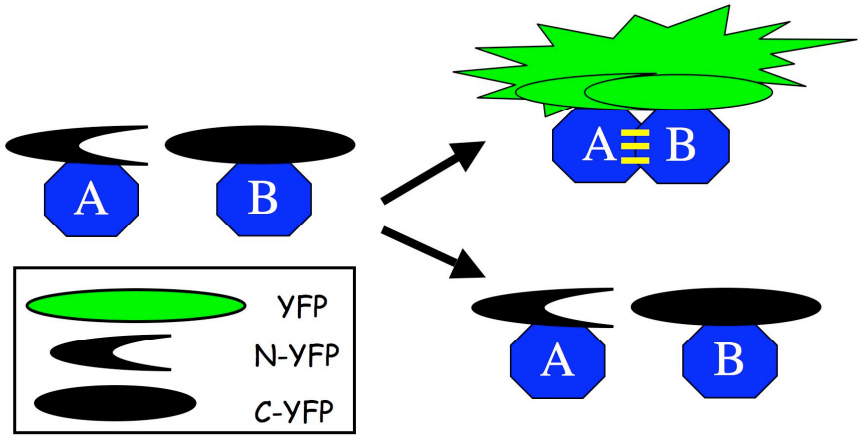


Figure R20. Schematic representation of Bimolecular Fluorescence Complementation assay used to detect interactions between two proteins. Proteins that are postulated to interact, here referred as A and B, are fused to unfolded complementary fragments of a fluorescent reporter protein and expressed in live cells. Interaction of these proteins will bring the fluorescent fragments within proximity, allowing the reporter protein to reconstitute and emit its fluorescent signal.

We used a simplification of the method involving just P1b fused to either of two fragments of the YFP (NYFP and CYFP), which are transiently expressed by agroinfiltration in *N. benthamiana* leaves (Fig. R21A). In order to have uniform high expression levels, agrobacteria expressing the TBSV P19 silencing suppressor were included in all the infiltration mixtures. No fluorescence was detected in cells expressing each P1b fusion product (p35S-NYFP-P1b or p35S-CYFP-P1b) independently or in combination with the complementary YFP fragment fused to a naïve protein (Fig. R21B and data not shown). In contrast, strong fluorescence was detected at 3 and 6 dpi under UV light in leaf patches coexpressing p35S-NYFP-P1b and p35S-CYFP-P1b (Fig. R21B and data not shown).

P1b mutants were also expressed as NYFP and CYFP fusion proteins and tested for interaction by BiFC. Mutations on the protease active center (S264A), the LxKA motif (RK68,69AA) and a non-conserved cysteine (C93A) did not affect the ability of P1b to self-interact, and fusion proteins with these mutations reconstituted fluorescent YFP with similar efficiency as wild type proteins (Fig. R21B). However, patches expressing P1b proteins with mutations at the zinc finger motif (C89A, C103A and C106A) displayed no fluorescence at 3 dpi (data not shown) and just a very weak signal at 6 dpi (Fig. R21B), suggesting that the predicted zinc finger plays an important role in P1b self-interaction *in vivo*.

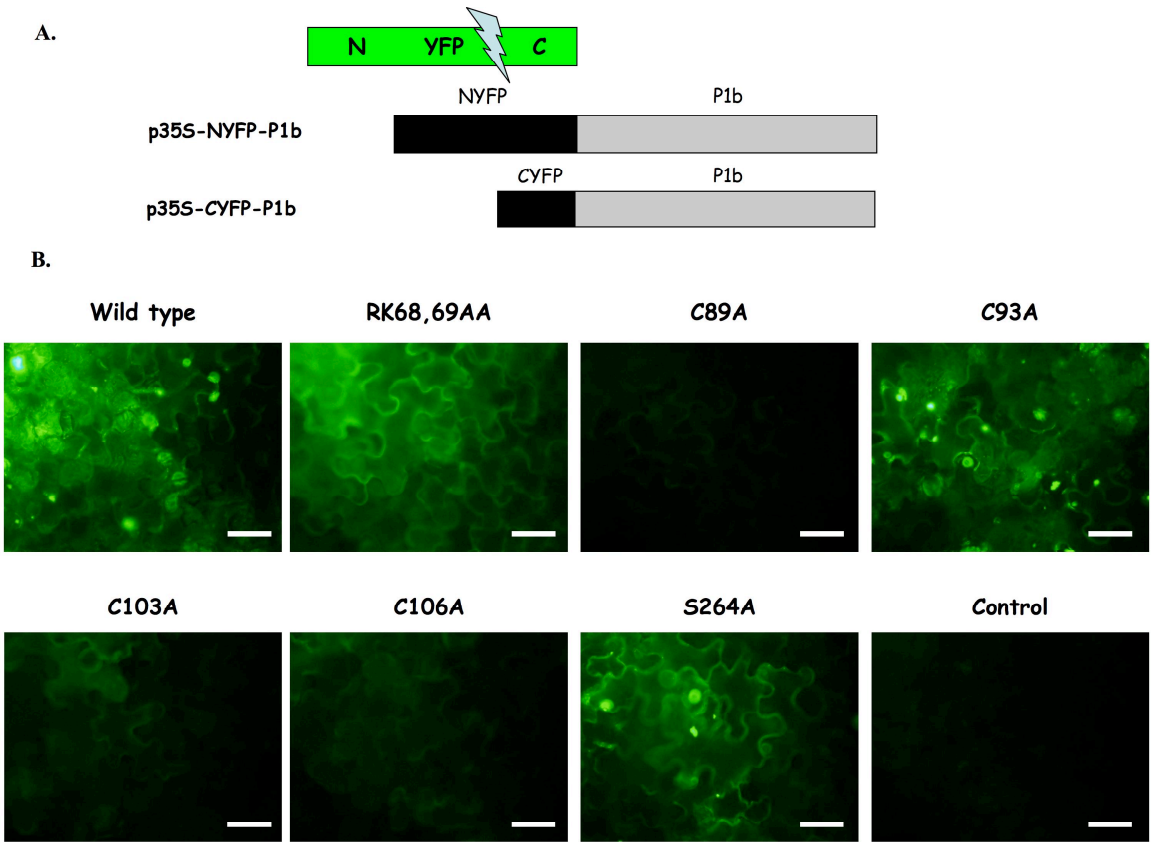


Figure R21. Analysis of CVYV P1b self-interaction by BiFC assay. *N. benthamiana* plants were coinfiltrated with agrobacteria carrying pBin61:P19 plus p35S-NYFP-P1b (wild type or the indicated mutants) plus p35S-CYFP-P1b (wild type or the indicated mutants). Plants coinfiltrated with agrobacteria carrying pBin61:P19 plus p35S-CYFP-P1b were used as negative control (Control). (A) Schematic representation of plasmids used in the assay. (B) YFP fluorescence pictures taken under a fluorescence microscope at 6 dpi. Scale bars, 50 µm.

III.3.4 P1b forms homodimers in solution

The self-interaction of P1b revealed by the BiFC assays suggested that this protein could form oligomeric structures. To test this possibility, we partially purified by affinity chromatography in calmoduline sepharose NTAP-P1b expressed in *N. benthamiana* leaves by agroinfiltration, and analyzed the purified protein by FPLC gel filtration. TAP-specific Western blot analysis of the collected fractions showed that NTAP-P1b migrated as a single peak close to a molecular mass marker of 158 kDa, which is much larger than the molecular mass of monomeric NTAP-P1b (58.4 kDa), suggesting that NTAP-P1b is in an oligomeric form (Fig. R22A). To rule out possible structural effects of the TAP tag, NTAP-P1b was trimmed by proteolytic processing with the TEV protease (Rohila *et al.*, 2004). This treatment removes the protein A domain of the TAP tag (15.4 kDa) leaving just the calmoduline binding protein domain (CBP, 6.8 kDa) fused to P1b (NCBP-P1b, 43 kDa). This sample was also analyzed by FPLC gel filtration. Western blot analysis with biotinylated calmoduline revealed that NCBP-P1b eluted as a single peak with an apparent molecular mass of ~100 kDa (Fig.

R22A), which is approximately double the predicted size for the NCBP-P1b monomer, further supporting the conclusion that P1b could acquire a homodimeric conformation.

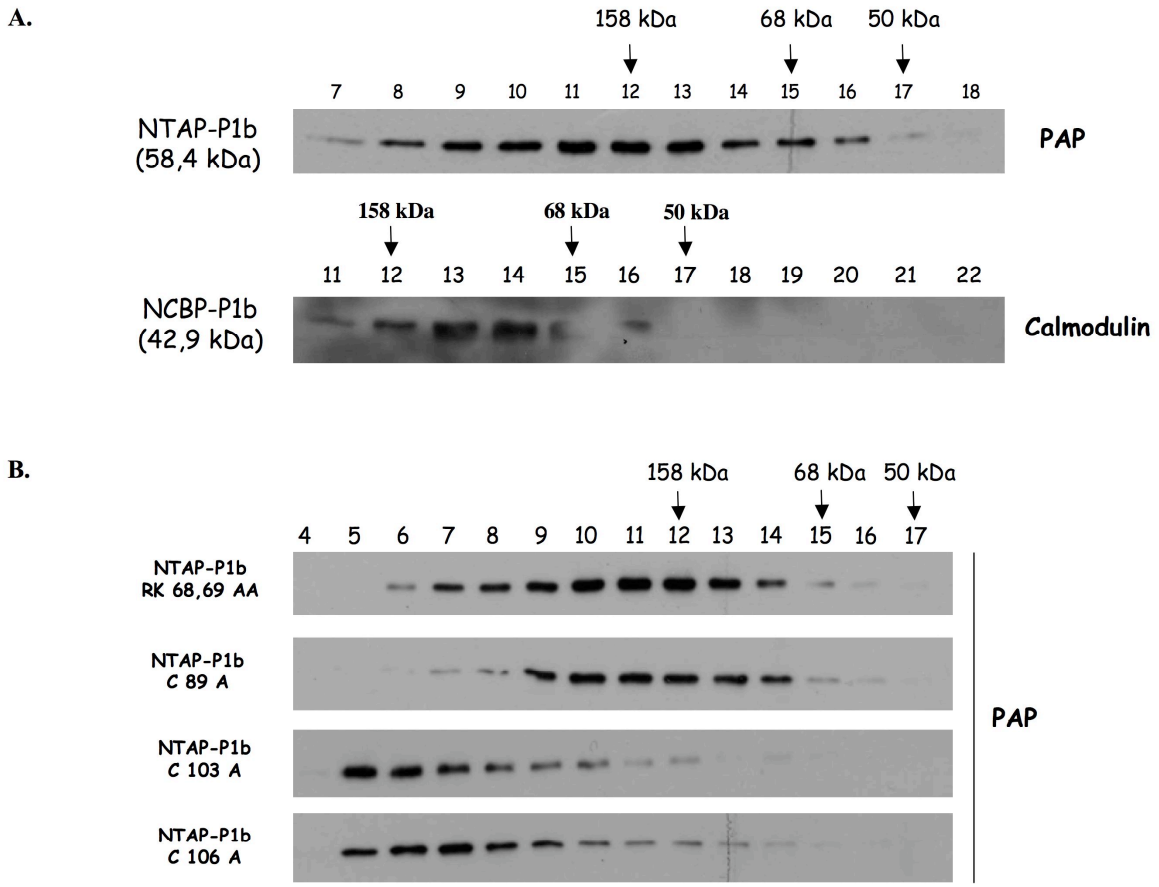


Figure R22. Oligomerization of CVYV P1b in solution. N-tagged CVYV P1b proteins purified by affinity chromatography were analyzed by gel filtration FPLC. (A) Elution fractions of NTAP-P1b intact or digested with TEV protease (NCBP-P1b) subjected to Western blot analysis with PAP complex or biotinylated calmoduline, respectively. (B) Elution fractions of NTAP-P1b mutants subjected to Western blot analysis with PAP complex. Arrows indicate the elution position of molecular mass markers: aldolase (158 kDa), serum albumin (68 kDa) and ovoalbumine (50 kDa).

NTAP-P1b mutants with defects in RNA silencing suppression activity were also analyzed by gel filtration FPLC (Fig. R22B). As expected, the NTAP-P1b RK68,69AA mutant, which was observed to self-interact *in vivo* in the BiFC assay, also appeared to homodimerize like the wild type protein (Fig. R22B). However, C103A and C106A mutations at the zinc finger motif, appeared to have a drastic effect on P1b conformation since NTAP-P1b with either of these mutations eluted in the first gel filtration FPLC fractions, indicative of non-specific aggregation (Fig. R22B). These data are consistent with the results of the BiFC assay and suggest a structural role for the predicted zinc finger. The gel filtration elution profile of NTAP-P1b C89A, which resembled the other zinc finger mutants in the inability to self-interact efficiently *in vivo* in the BiFC assay, was usually similar to those of wild type

NTAP-P1b and the RK68,69AA mutant (Fig. R22B), although some NTAP-P1b C89A non-specific aggregation was also observed in some purification experiments (data not shown).

III.3.5 P1b is a siRNA binding protein

CVYV P1b closely resembled P1-HCPro of the potyvirus PPV in different RNA silencing suppression assays, suggesting that both viral suppressors could target the same step(s) of the silencing pathway. Although the mechanism that potyviral HCPro uses to interfere with RNA silencing has still not been completely unravelled, the activity of HCPro, as well as that of tombusvirus P19 and other silencing suppressors, involves direct sequestering of double-stranded siRNAs (Lakatos *et al.*, 2006; Mérai *et al.*, 2006). The possibility that siRNA binding could also play a role in the RNA silencing suppression activity of P1b was assessed by electrophoretic mobility shift assays (EMSA). Crude extracts from *N. benthamiana* leaves agroinfiltrated with p35S-NTAP-P1b or pBIN61:P19, which expresses TBSV P19 and was used as a positive control, were incubated with ^{32}P-labelled synthetic double stranded siRNAs, and the resulting complexes were resolved by gel electrophoresis (Fig. R23A). As expected, P19 caused a shift in siRNA mobility. Interestingly, an siRNA complex of lower mobility than the P19-siRNA complex was formed by the NTAP-P1b extract, suggesting that NTAP-P1b may interact with siRNAs or induce siRNA interaction with another protein of the plant extract. To discriminate between these two possibilities, we carried out a super shift assay using PAP complex, which interacts specifically with TAP. PAP had no effect on the mobility of free siRNA or of P19-siRNA complexes, but caused a further band shift of siRNAs complexes formed by the NTAP-P1b extract, indicating that NTAP-P1b was a component of these siRNA complexes (Fig. R23A).

To further confirm the NTAP-P1b/siRNA interaction, NTAP-P1b partially purified by affinity chromatography was subjected to the EMSA assay (Fig. R23B). Purified NTAP-P1b formed an siRNA complex of the same mobility than that formed by the crude extracts of leaves expressing NTAP-P1b. This complex also suffered a super shift when it was incubated with the PAP reagent (Fig. R23B).

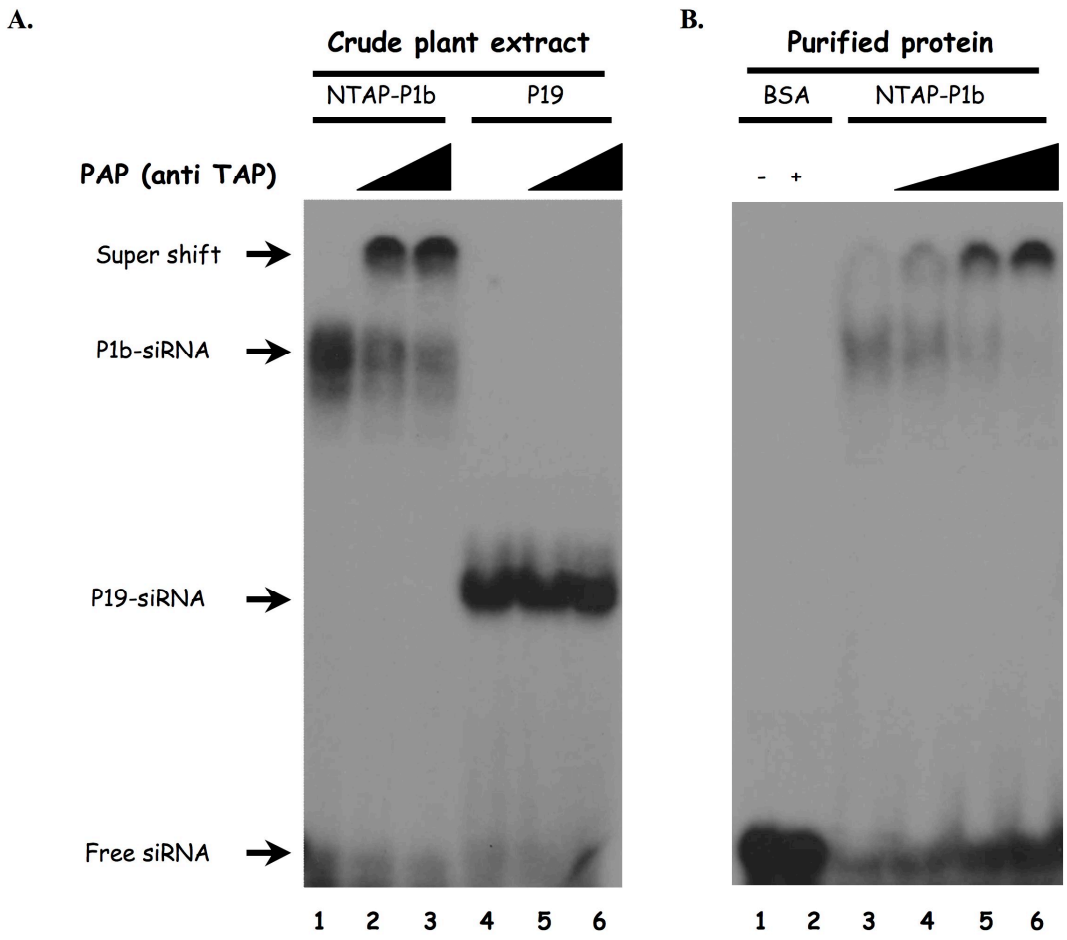


Figure R23. CVYV P1b binds siRNA *in vitro*. (A) Crude protein extract (2 µl) from agroinfiltrated leaves expressing NTAP-P1b (lanes 1-3) or TBSV P19 (lanes 2-5) harvested at 3 dpi were incubated with ^{32}P-labelled double stranded siRNAs. PAP complex was included in the binding mixtures loaded in lanes 2 and 5 (0.1 µl), and 3 and 6 (0.2 µl). (B) NTAP-P1b purified by affinity chromatography (250 nM) (lanes 3-6) or control serum albumin (250 nM) (lanes 1-2) were incubated with siRNAs. PAP complex was included in the binding mixtures loaded in lanes 4 (0.02 µl), 5 (0.1 µl) and 2 and 6 (0.2 µl). Protein-siRNA complexes were resolved in polyacrylamide gels and revealed by autoradiography.

Crude extracts of leaves agroinfiltrated with plasmids expressing NTAP-tagged P1b mutants were also subjected to EMSA assays (Fig. R24). Whereas C93A and S264A mutants, which are active silencing suppressors, bound siRNAs like wild type P1b, no siRNA binding was detected for any inactive mutant even when the dose of extract was increased to have an excess of mutant protein with respect to wild type P1b (Fig. R24). These results demonstrate that P1b rather than the TAP tag is responsible for siRNA binding of NTAP-P1b, and highlight the relevance of this interaction for the RNA silencing suppression activity of P1b.

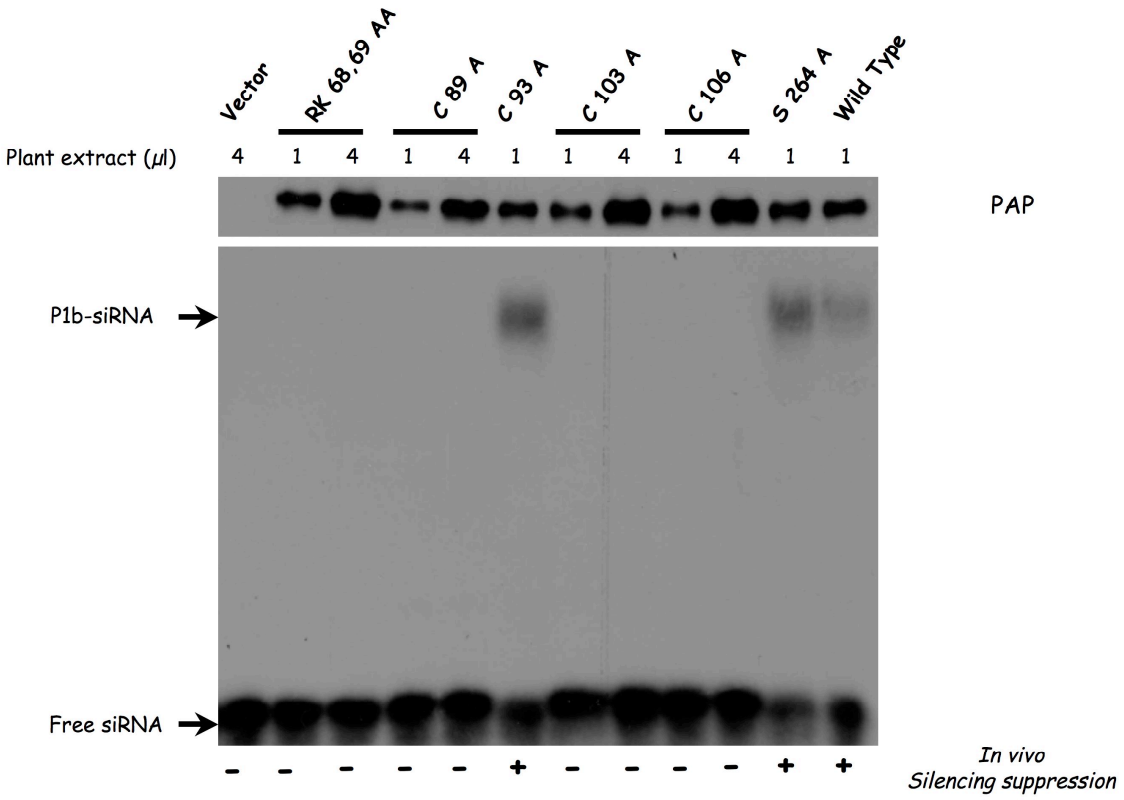


Figure R24. Null-silencing suppression CVYV P1b mutants are unable to bind siRNAs. Crude protein extract (1 or 4 µl) from leaves infiltrated with agrobacteria carrying p35S-NTAP-P1b (wild type or the indicated mutants) or empty pBin19 (vector) harvested at 3 dpi were incubated with ^{32}P-labelled double stranded siRNAs. Protein-siRNA complexes were resolved in polyacrylamide gels and revealed by autoradiography. The amount of NTAP-P1b protein present in the crude extracts was estimated by Western blot analysis with PAP complex (top panel). The silencing suppression activity of each NTAP-P1b mutant is indicated at the bottom.

III.4 The specific binding to 21-nt double stranded RNAs is crucial for the anti-silencing activity of CVYV P1b and perturbs endogenous small RNA populations

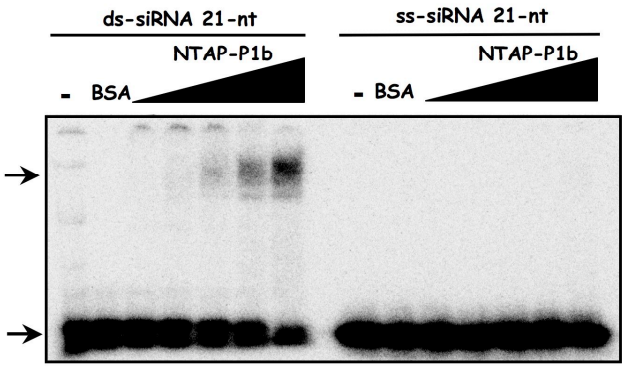
The fact that CVYV P1b is able to bind *in vitro* siRNAs, key elements of the RNA silencing machinery, suggests that siRNA sequestration plays a major role in the suppression activity of this protein, as it has been demonstrated or suggested for other RSSs (Lakatos *et al.*, 2006; Vargason *et al.*, 2003).

In the present chapter, I show that CVYV P1b binds small RNAs *in vivo* and causes a drastic disturbance in the endogenous populations of these molecules, and that the ability to bind small RNAs of CVYV P1b is precisely correlated with its silencing suppression activity. In addition, the reported data show specific features of the small RNA molecules relevant for CVYV P1b recognition, which only partially match those contributing to potyviral HCPro binding, and map a CVYV P1b siRNA-binding domain.

III.4.1 CVYV P1b binds preferentially double strand siRNAs of 21 nt

In vitro results described in the previous chapter show that the P1b protein of CVYV resembles the typical potyviral RSS HCPro in its ability to interact with siRNAs (see section III.3.5). To further characterize this activity of CVYV P1b, its binding to different nucleic acid molecules was assayed by EMSA. TAP-tagged P1b was expressed by agro-infiltration in *N. benthamiana* plants, purified by affinity chromatography, and then probed with either single-stranded (ss-) or double-stranded (ds-) ^{32}P-labeled siRNAs. As expected, NTAP-P1b caused a shift in the electrophoretic mobility of 21-nt ds-siRNA. In contrast, NTAP-P1b did not affect the mobility of 21-nt ss-siRNA (Fig. R25A). In addition, NTAP-P1b was also unable to form low-mobility complexes with 21-nt DNA molecules regardless they were ss- or ds- (Fig. R25B). These data show that P1b is a nucleic acid binding protein with strong preferences for ds-RNA.

A.



B.

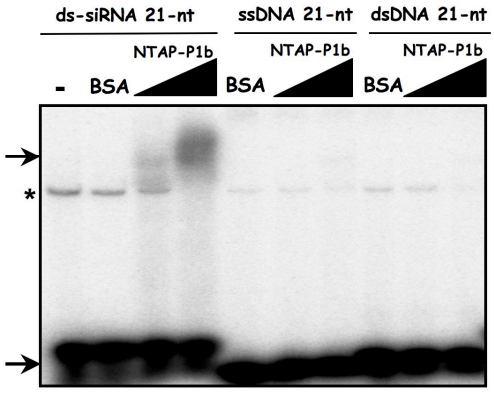


Figure R25. P1b binds specifically ds-siRNAs. (A) Increasing amounts of NTAP-P1b purified by affinity chromatography (30, 60, 120, 240 and 480 nM), as well as bovine serum albumin (BSA; 250 nM) or just buffer (-), were incubated with the indicated ^{32}P-labeled small RNAs. (B) Increasing amounts of NTAP-P1b purified by affinity chromatography (100 and 300 nM), as well as BSA (500 nM) or just buffer (-), were incubated with the indicated ^{32}P-labeled nucleic acids. Double stranded molecules had 2-nt 3' protruding ends. Complexes were resolved in polyacrilamide gels and revealed by autoradiography. The arrows indicate bound and free ^{32}P-labeled probes. The asterisk indicates a non-specific shift.

Double-stranded RNA binding appears to be a usual strategy of plant virus RSSs to counteract the silencing machinery (Mérai *et al.*, 2006); however, they may differ in RNA size selectivity, thus causing specific effects in different steps of the silencing pathways (Baulcombe & Molnár, 2004). To test whether P1b can discriminate between RNAs of different sizes, we carried out direct competition assays using as source of suppressor protein crude extracts of agroinfiltrated *N. benthamiana* leaves expressing NTAP-P1b. A fixed amount of ^{32}P-labeled 21-nt ds-siRNAs was mixed with increasing amounts of 21-, 24- or 26-nt unlabeled ds-siRNAs and incubated with leaf extracts. The resulting complexes were then resolved by gel electrophoresis (Fig. R26A). Although unlabeled 24-nt and, to lesser extent, 26-nt ds-siRNAs were able to compete the binding of the ^{32}P-labeled 21-nt ds-siRNAs to NTAP-P1b, they prevented the binding of the radioactive probe much less efficiently than the

21-nt ds-siRNA competitor, and they were unable to produce a complete competition even with a 2560-fold molar excess (Fig. R26B). This result suggests that P1b binds preferently ds-siRNAs of 21 nt, the main siRNA species produced by RNA silencing from plant RNA viruses.

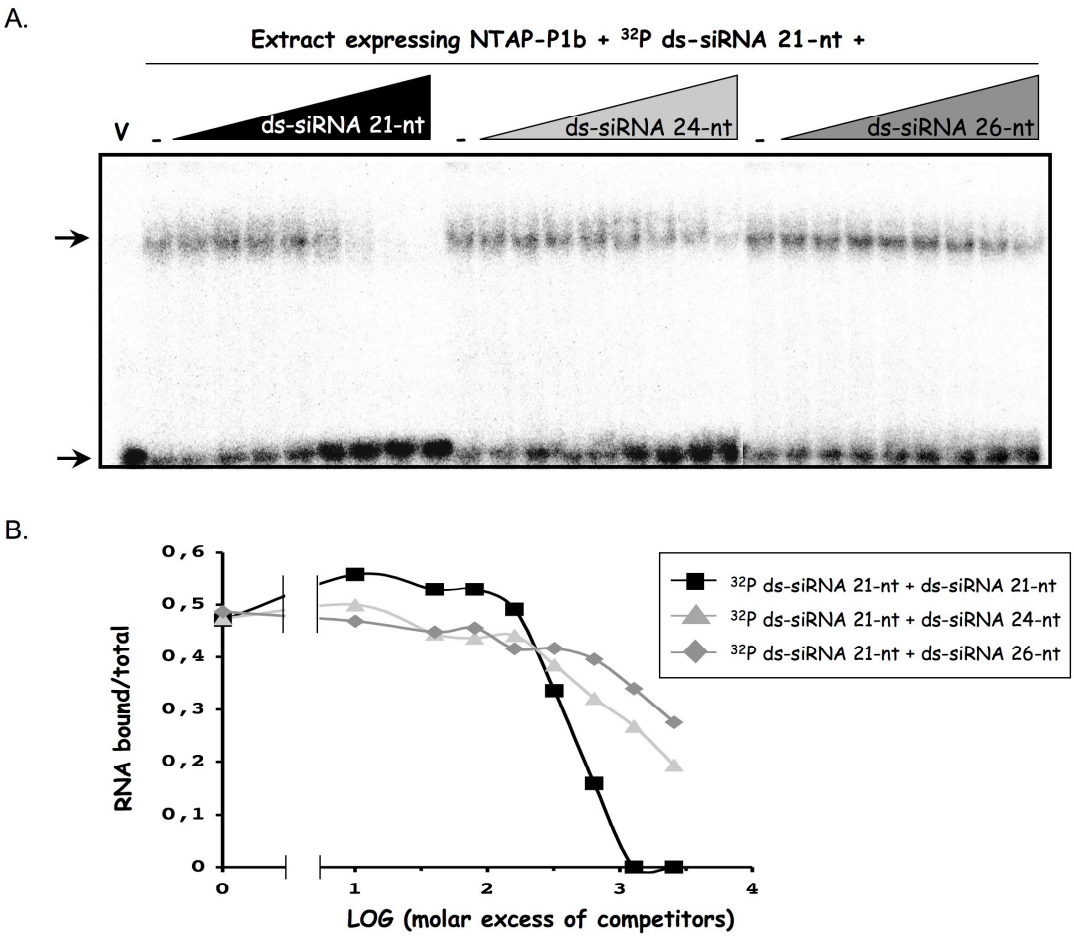


Figure R26. P1b binds ds-siRNAs with size selectivity. (A) Crude protein extracts from *N. benthamiana* leaves infiltrated with agrobacteria carrying p35S-NTAP-P1b or the empty pBIN19 vector (lane V), and harvested at 6 dpi, were incubated with ^{32}P-labeled 21-nt ds-siRNAs in the presence of the indicated unlabeled ds-siRNA competitors added in increasing molar excess (10-, 40-, 80-, 160-, 320-, 640-, 1280- and 2560-fold) or in the absence of them (lanes V and -). Complexes were resolved in polyacrilamide gels and revealed by autoradiography. Arrows indicate bound and free ^{32}P-labeled 21-nt ds-siRNAs. (B) Densitometric analysis of the autoradiographic signals. The ratio bound RNA/total RNA of each lane was plotted as a function of logarithm of molar excess of competitors. All ds-siRNAs had 2-nt 3' protruding ends.

III.4.2 P1b and HCPro show different structural requirements for siRNA binding

Natural siRNAs have specific structural features, namely a 5' terminal phosphate and a 2-nt overhang with a free OH at the 3' end, which result from the cleavage of any Dicer-like (DCL) enzyme. These features can be specifically recognized by different siRNA-binding proteins (Ma *et al.*, 2004; Mérai *et al.*, 2006; Nykänen *et al.*, 2001). For instance, HCPro, the main RSS from potyviruses, shows a higher affinity for 21-nt ds-siRNAs carrying the 3' overhang than by the equivalent 19-nt blunt-ended ds-siRNA (Lakatos *et al.*, 2006; Mérai *et*

al., 2006). In order to ascertain whether CVYV P1b also requires the 2-nt 3' overhang for efficient binding, protein extracts of agroinfiltrated *N. benthamiana* leaves expressing either NTAP-P1b or the HCPro of the potyvirus PPV were used for EMSA competition assays. Extracts were incubated with ^{32}P-labeled 21-nt ds-siRNAs with 3' overhangs and increasing amounts of unlabeled competitors: either 21-nt siRNAs with 3' overhangs or 19-nt blunt ended siRNAs, and the resulting complexes were resolved by gel electrophoresis. NTAP-P1b showed in this assay slight preference for 19-nt ds-siRNAs (Fig. R27A), whereas PPV HCPro interacted preferentially with 21-nt ds-siRNAs (Fig. R27B), as it has been previously reported for the HCPro from another potyvirus, TEV (Lakatos *et al.*, 2006; Mérai *et al.*, 2006).

Intriguingly, the excess of unlabeled competitors required to displace the ^{32}P-labeled siRNA was much higher for HCPro than for NTAP-P1b (Fig. R27A and B). Having in mind that unlabeled competitors used in these experiments have a free 5'OH and that a 5' phosphate could be relevant for siRNA recognition (Vargason *et al.*, 2003), the different competition efficiency for NTAP-P1b and HCPro siRNA bindings could be reflecting different requirements of these two potyviral RSSs for specific 5' terminal structures. To assess this possibility, extracts of agroinfiltrated *N. benthamiana* leaves expressing either PPV HCPro or CVYV NTAP-P1b were incubated with ^{32}P-labeled 21-nt ds-siRNAs and increasing amounts of equivalent 21-nt ds-siRNAs with or without 5' P groups, and the resulting protein-siRNA complexes were resolved by gel electrophoresis. In agreement with the result of the previous experiment, whereas NTAP-P1b bound 5'-P and 5'-OH siRNAs species with similar affinity (Fig. R27C), PPV HCPro showed a strong predilection for siRNA carrying 5' P group (Fig. R27D).

Thus, in spite of the very similar behaviour shown by potyviral HCPro and CVYV P1b in different functional assays, and of their shared ability to bind ds-siRNAs of 21 nt, these two phylogenetically unrelated potyviral RSSs recognize siRNAs with some specific structural requirements. In addition, our findings point to a rather specific mechanism of nucleic acid binding mediated by CVYV P1b, since it differs from the well studied RSSs tombusviral P19 and potyviral HCPro in binding siRNAs without apparent structural constraints at both siRNA ends.

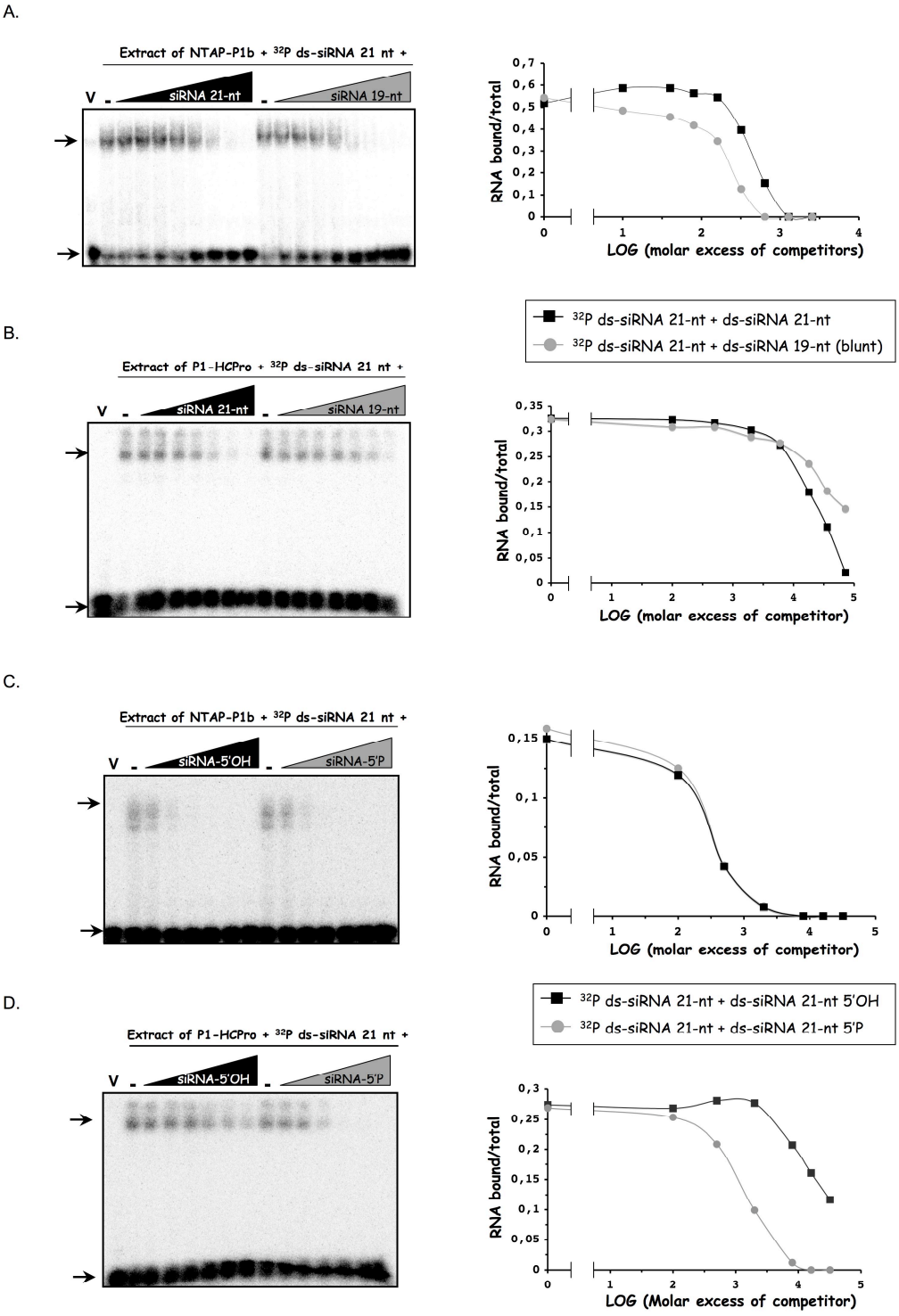


Figure R27. P1b and HCPro display different structural requirements for efficient siRNA recognition. Crude protein extracts from *N. benthamiana* leaves infiltrated with agrobacteria carrying p35S-NTAP-P1b (A and C), p35S-P1HC (B and D) or the empty pBIN19 vector (lanes V), and harvested at 6 dpi, were incubated with ^{32}P-labeled 21-nt ds-siRNAs in the absence (lanes V and -) or in the presence of increasing molar excesses (A,10-2560-fold; B, 100-72000-fold; C and D, 100-32000-fold) of unlabeled competitors: non-phosphorylated 21-nt ds-siRNAs with 2-nt 3' protruding ends (A-D); non-phosphorylated blunt-ended 19-nt ds-siRNAs (A and B); phosphorylated 21-nt ds-siRNAs with 2-nt 3' protruding ends (C and D). Complexes were resolved in polyacrilamide gels and revealed by autoradiography (left panels). Arrows indicate bound and free ^{32}P-labeled 21-nt ds-siRNAs. For all the competition experiments, densitometric analyses of the autoradiographic signals are shown at the right panels. The ratio bound RNA/total RNA of each lane was plotted as a function of logarithm of molar excess of competitors.

III.4.3 CVYV P1b binds siRNA in vivo

The fact that CVYV P1b binds ds-siRNAs *in vitro* with size specificity supports the idea that it could counteract RNA silencing by interaction and sequestering of these molecules *in vivo*. To test this hypothesis, a dsRNA-triggered silencing suppression assay coupled with *in vivo* siRNA pull-down was carried out. Thus, *N. benthamiana* leaves were agro-infiltrated with a mixture of p35S:GFP (expressing GFP mRNA as reporter) and the strong silencing inductor p35S:GF-IR (expressing an inverted repeat which generates a partial GFP dsRNA) plus a plasmid expressing either wild type NTAP-P1b or its RK68,69AA inactive mutant (see chapter III.3).

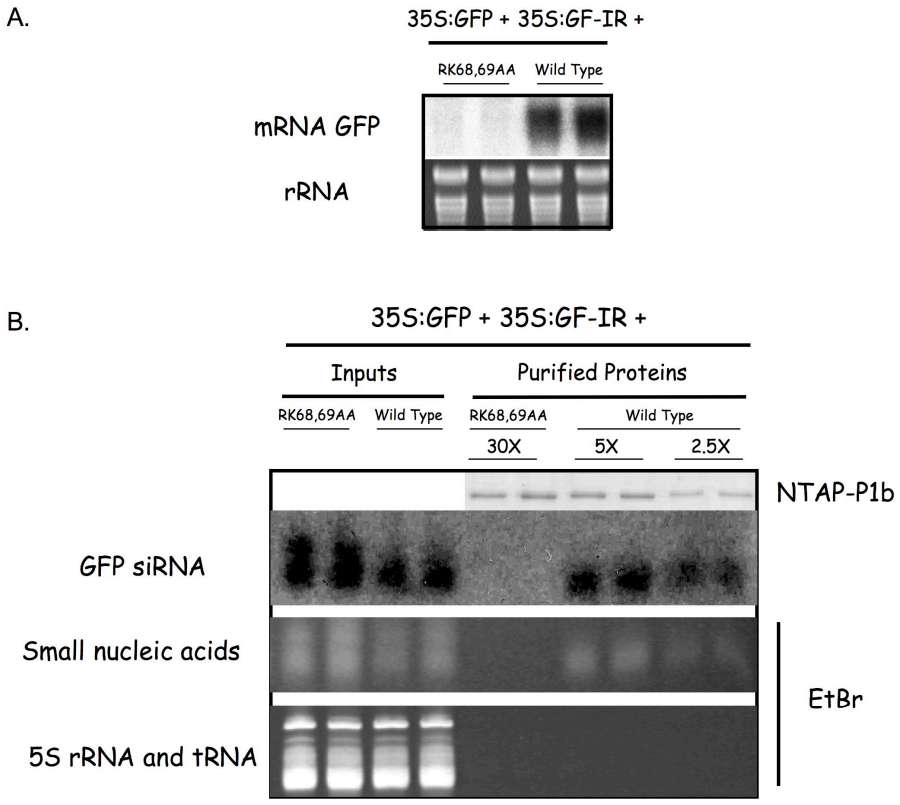


Figure R28. CVYV P1b binds siRNAs *in vivo*. *N. benthamiana* plants were co-infiltrated with agrobacteria carrying p35S:GFP and p35S:GF-IR plus p35S-NTAP-P1b or p35S-NTAP-P1b RK68,69AA. The infiltrated leaves were harvested at 6 dpi. (A) Northern blot analysis of GFP mRNA extracted from infiltrated leaves. Agarose gel stained with BrEt is shown as loading control (rRNA). (B) Small RNAs separated by polyacrylamide gel electrophoresis and stained with BrEt (Small nucleic acids) and GFP-specific siRNAs detected by Northern blot analysis (GFP siRNAs) from either infiltrated leaves (Inputs) or NTAP-P1b, wild type or RK68,69AA mutant, purified by affinity chromatography (Purified Proteins). 5S rRNA and tRNA stained with BrEt are shown as a loading control. The samples of purified NTAP-P1b proteins used for the siRNA extraction were subjected to SDS-polyacrylamide gel electrophoresis to assess their protein amounts (NTAP-P1b).

As expected, Northern blot analysis showed that the co-infiltration with wild type p35S-NTAP-P1b prevented GFP mRNA silencing, whereas co-infiltration with p35S-NTAP-P1b RK68,69AA failed to prevent reporter degradation (Fig. R28A). Similar levels of GFP-

specific siRNAs were detected in total extracts of agroinfiltrated leaves regardless they expressed the active silencing suppressor or the inactive mutant. However, after purification of the TAP-tagged proteins, Northern blot analysis detected GFP-derived siRNAs in the affinity chromatography eluate of wild type NTAP-P1b, but not in that of NTAP-P1b RK68,69AA (Fig. R28B). In addition, the presence of small nucleic acids was revealed by BrEt staining in the sample corresponding to purified wild type NTAP-P1b, but not in the equivalent RK68,69AA sample (Fig. R28B, lower panel). Together, these results indicate that CVYV P1b interacts with siRNAs *in planta*, and that this ability is prevented by the RK68,69A mutation.

III.4.4 CVYV P1b induces drastic changes in N. benthamiana small RNA populations

The ability of CVYV P1b to interact with small RNAs *in vivo* prompts the suggestion that expression of this RSS in a plant could cause alterations in its endogenous populations of small RNAs. In addition, size-specific effects are expected in line with the size-selectivity of the siRNA binding observed in the *in vitro* assays (Fig. R26). To test these assumptions and to extensively scrutinize the P1b binding preferences *in vivo*, a deep sequencing analysis of different samples of *N. benthamiana* small RNAs was carried out. Small RNAs purified from crude extracts of untreated leaves (Nb) or of leaves infiltrated with an *Agrobacterium* strain expressing NTAP-P1b (Nb+P1b), and a small RNA fraction co-purified with NTAP-P1b by affinity chromatography from the agroinfiltrated leaves (CoP-P1b), were amplified by RT-PCR and sequenced by the Solexa-Illumina system. The effect of P1b on exogenous siRNAs derived from the agroinfiltrated p35S-NTAP-P1b plasmid and on endogenous *N. benthamiana* small RNAs were analyzed separately (Fig. R29A). The small RNAs from *N. benthamiana* leaves were enriched, by far, in 24-nt species displaying also a modest peak of 21-nt siRNAs (Fig. R29B, left panel). In contrast, when NTAP-P1b was expressed in leaf tissues, a shift on the small RNAs size profile was observed (Fig. R29B, middle panel). For agroinfiltrated leaves expressing NTAP-P1b, the main peak corresponded to 21-nt species, with moderate accumulations of 22- and 24-nt small RNA molecules (Fig. R29B, middle panel). These changes on size profile were not observed when an empty binary vector was agro-infiltrated (data not shown). Interestingly, NTAP-P1b protein co-purified with a population of endogenous small RNAs of mainly 21 nt in length, with very little representation of 23- and 24-nt species (Fig. R29B, right panel).

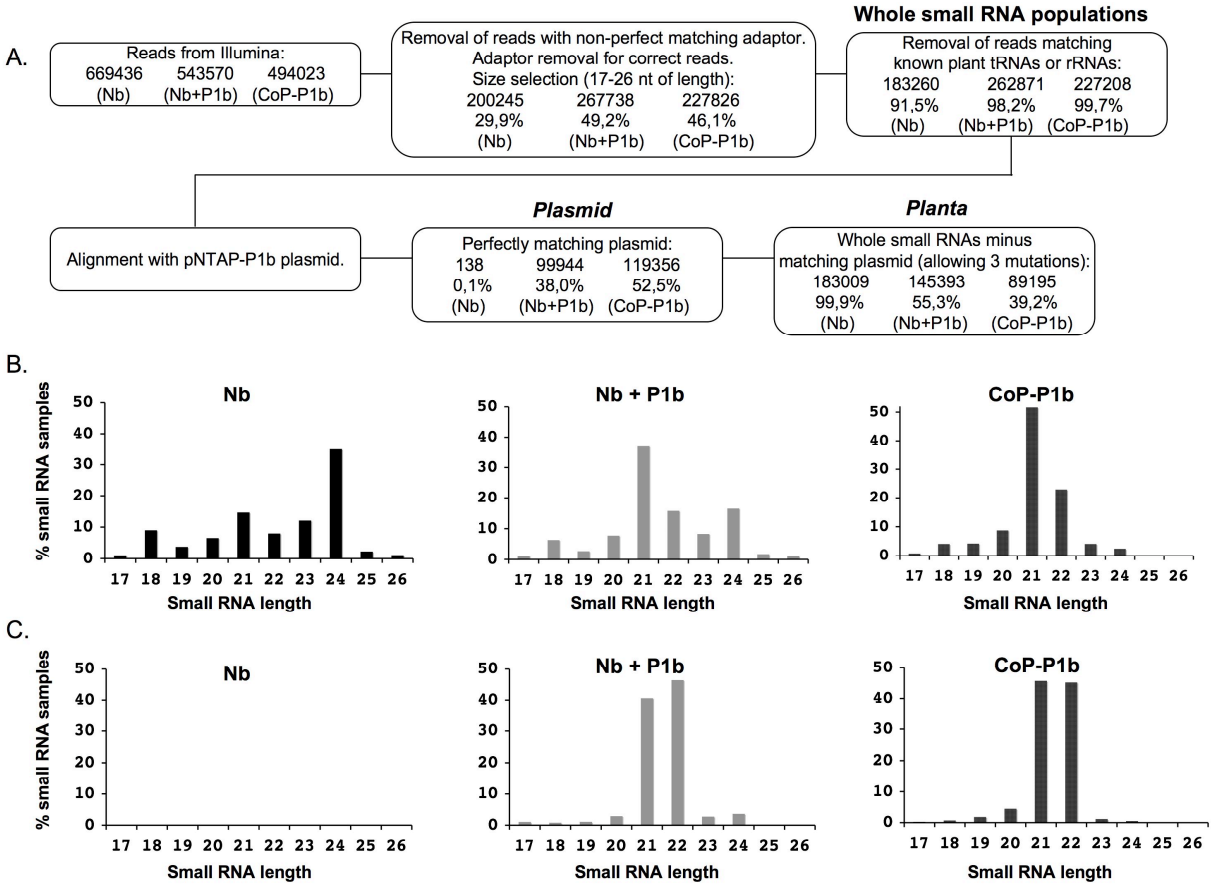


Figure R29. Enrichment in 21-nt small RNAs associated to NTAP-P1b expression. (A) Flowchart of the analysis of small RNA sequences obtained by deep sequencing of small RNAs from *N. benthamiana* (Nb), *N. benthamiana* infiltrated with agrobacteria carrying p35S-NTAP-P1b (Nb+P1b), or a fraction of NTAP-P1b purified by affinity chromatography from these plants (CoP-P1b). The number of reads passing each step and the percentage respect to the previous step are shown for each sample. (B) Size-distribution histograms of endogenous (*planta*) small RNAs of each sample, expressed as percentage respect to the *planta* small RNA population. (C) Size-distribution histograms of plasmid-derived (*plasmid)* small RNAs of each sample, expressed as percentage respect to the *plasmid* small RNA population.

Segregated populations of small RNAs matching the p35S-NTAP-P1b sequence were also analyzed. As expected, whereas the non-agroinfiltrated Nb sample had no plasmid-derived siRNAs, a relative high amount of these molecules were found in both Nb+P1b and CoP-P1b samples (38% and 52.5% of whole small RNAs, respectively; Fig. R29A). The size profile analysis showed that, in both cases, most of species were 21 or 22 nt in length, although the 21-nt molecules appeared to be slightly overrepresented in the siRNA population copurified with NTAP-P1b (Fig. R29C). It is also remarkable that the accumulation of siRNAs of 24 nt, which was very low in the sample from leaves expressing NTAP-P1b, was even more reduced in the sample of NTAP-P1b-bound siRNAs.

All together, the deep sequencing analysis confirmed that P1b binds endogenous small RNAs with a high preference for 21- and 22-nt species, and that this RSS causes a strong rearrangement of the plant small RNA populations.

III.4.5 A basic conserved domain is involved in small RNA binding

In silico predictions failed to identify a canonical dsRNA binding domain in CVYV P1b, however the results shown in the chapter III.3 suggested that a basic LxKA motif, which is partially conserved in P1b-like proteins, could be involved in siRNA binding. In order to confirm this hypothesis, point mutations affecting basic amino acids of this region were introduced in p35S-NTAP-P1b, and the silencing suppression and siRNA-binding activities of the mutated proteins were assayed.

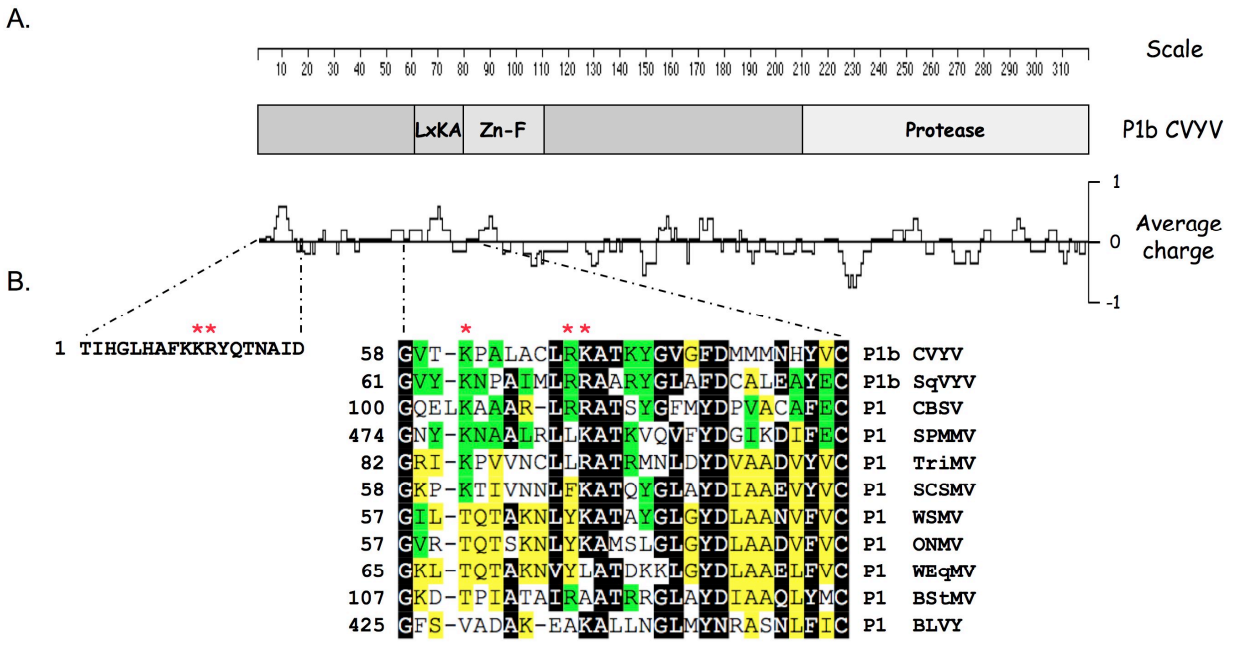


Figure R30. Basic domains in P1b-like proteins. (A) Schematic representation of CVYV P1b showing the location of conserved domains and a plot of charge density along the protein. (B) Details of two basic domains placed at the N-terminal half of CVYV P1b. An amino acid alignment of the second domain, which is partially conserved in P1b-like proteins, is shown. Proteins included in the alignment are: P1b from the ipomoviruses CVYV and SqVYV, and P1 from the ipomoviruses CBSV and SPMMV, from the tritimoviruses WSMV, ONMV, WeqMV and BStMV, and from the still unclassified potyvirid TriMV, SCSMV and BLVY. Boxed amino acids are identical or chemically similar between at least two ipomoviruses (green boxes), between at least three non-ipomoviruses (yellow boxes), or between at least two ipomoviruses plus two non-ipomoviruses (black boxes). Dashes represent gaps. The position of the first amino acid of each aligned segment is indicated on the left size of the sequence. The asterisks indicate the residues of CVYV P1b that were mutated in this work.

We also analyzed the effects on these activities of mutations affecting amino acids of a non-conserved basic region present at the N-terminus of the CVYV P1b protein (Fig. R30). A double mutant in this domain, KR10,11AA, showed a silencing suppressing activity indistinguishable from that of wild type NTAP-P1b (Fig. R31A and B). In addition, the siRNA binding activity of NTAP-P1b KR10,11AA was comparable to that of wild type protein in an EMSA *in vitro* test.

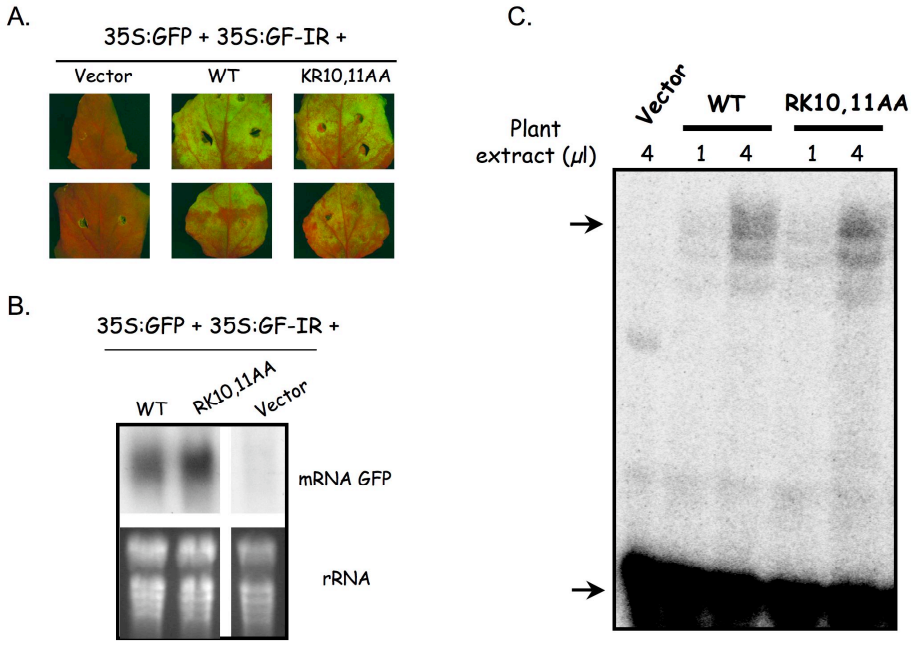


Figure R31. Silencing suppression activity and siRNA binding capacity of P1b RK10,11AA mutant protein. *N. benthamiana* plants were coinfiltrated with agrobacteria carrying p35S:GFP and p35S:GF-IR plus empty pBIN19 plasmid (Vector), wild type p35S-NTAP-P1b or derivative of this plasmid with the KR10,11AA mutation. The infiltrated leaves were harvested at 4 dpi. (A) GFP fluorescence pictures taken under a fluorescence stereomicroscope. (B) Northern blot analysis of GFP mRNA extracted from infiltrated leaves. Agarose gel stained with BrEt is shown as loading control (rRNA). (C) siRNA binding analysis by EMSA. Crude protein extracts of infiltrated leaves (two doses: 1 and 4 µl) were incubated with ^{32}P-labeled 21-nt ds-siRNAs. Complexes were resolved by polyacrilamide gel electrophoresis and revealed by autoradiography. Arrows indicate bound and free ^{32}P-labeled 21-nt ds-siRNAs.

Results shown above indicated that the RK68,69AA mutation at the LxKA motif impairs both siRNA binding (Figs. R24 and R28) and silencing suppression activities (Figs. R18 and R19) of P1b, without perturbing its ability to self-interact (Figs. R21 and R22). We made now two single mutations changing independently arginine 68 and lysine 69 to alanine. In addition, arginine 68 was also replaced by leucine, the residue present at this position in the P1 protein of SPMMV, the type member of the ipomovirus genus, and another basic residue, lysine 61 was replaced by alanine (Fig. R30). The effects of these amino acid changes on RNA silencing suppression activity were assessed in a dsRNA-triggered agroinfiltration assay. In *N. benthamiana* leaves expressing p35S:GFP and p35S:GF-IR plus an empty vector, GF-IR directed strong silencing of the GFP reporter gene, and as consequence, no green fluorescence was detected in the infiltrated patches (Fig. R32A), and very low accumulation of GFP mRNA was detected by Northern analysis (Fig. R32B). In contrast, co-agroinfiltration of p35S:GFP, p35S:GF-IR, and wild type p35S-NTAP-P1b prevented mRNA GFP degradation, and strong green fluorescence (Fig. R32A) and high levels of GFP mRNA (Fig. R32B) were observed. Mutations K61A, R68A and K69A, as the previously reported RK68,68AA mutation, had a drastic effect on the silencing suppression activity of NTAP-

P1b, since green fluorescence was not observed in leaves in which NTAP-P1b carrying these mutations were expressed along with GFP mRNA and GFP-IR (Fig. R32A). Levels of accumulation of GFP mRNA were very low in these leaves, although those of the leaves expressing NTAP-P1b K69A appeared to be slightly higher than those of the leaves expressing K61A, R68A, RK68,69AA, or the empty vector (Fig. R32B). Interestingly, the R68L mutation had a very low effect on silencing suppression activity, and both green fluorescence (Fig. R32A) and GFP mRNA accumulation (Fig. R32B) of leaves expressing NTAP-P1b R68L were very similar to those of leaves expressing wild type NTAP-P1b.

None of the NTAP-P1b mutant proteins lacking silencing suppression activity was able to bind siRNAs in an EMSA test (Fig. R32C). Since a bi-molecular complementation assay showed that NTAP-P1b K61A maintains the self-interaction ability (data not shown), as it was also shown for the double mutant RK68,69AA (see section III.3.3), the inability to suppress silencing of the K61A, R68A and K69A variants appears to be the direct consequence of a defect in siRNA binding.

Surprisingly, the R68L mutant, which displayed a strong silencing suppression activity (Fig. R32A and B), was completely unable to interact with siRNAs in the *in vitro* EMSA test (Fig. R32C) challenging the hypothesis that siRNA binding is required for the silencing suppression activity of CVYV P1b. To verify this conflicting result, an *in vivo* siRNA pull-down was carried out. TAP-tagged proteins were purified from agro-infiltrated leaves by affinity chromatography, and the presence of siRNAs derived from the GFP sequence included in the IR trigger (GF) and of secondary siRNAs derived from the 3' end of the reporter mRNA (P) was tested by Northern blot analysis (Fig. R32D). The accumulations of GF siRNAs were similar in all infiltrated leaves (Fig R32D, input). However, leaves expressing active versions of NTAP-P1b (wild type and R68L) showed lower accumulations of P siRNAs in comparison with those expressing inactive mutants (Fig. R32D, input). As expected, GF and P siRNAs of approximately 21 nt copurified with wild type NTAP-P1b. Interestingly, in contrast with the EMSA results, all mutant proteins, except RK68,69AA, pulled GFP siRNAs down, although with different efficiencies (Fig. R32D, pull down). In agreement with its high silencing suppression activity, NTAP-P1b R68L was the mutant protein that captured the largest amount of siRNAs, but this was lower than that copurified with the wild type protein. NTAP-P1b K69A, which slightly protected GFP mRNA from degradation (Fig. R32B), was more effective in siRNA pull down than the K61A and R68A versions, which appear to be as inactive as RK68,69AA in suppressing the RNA silencing (Fig. R32B).

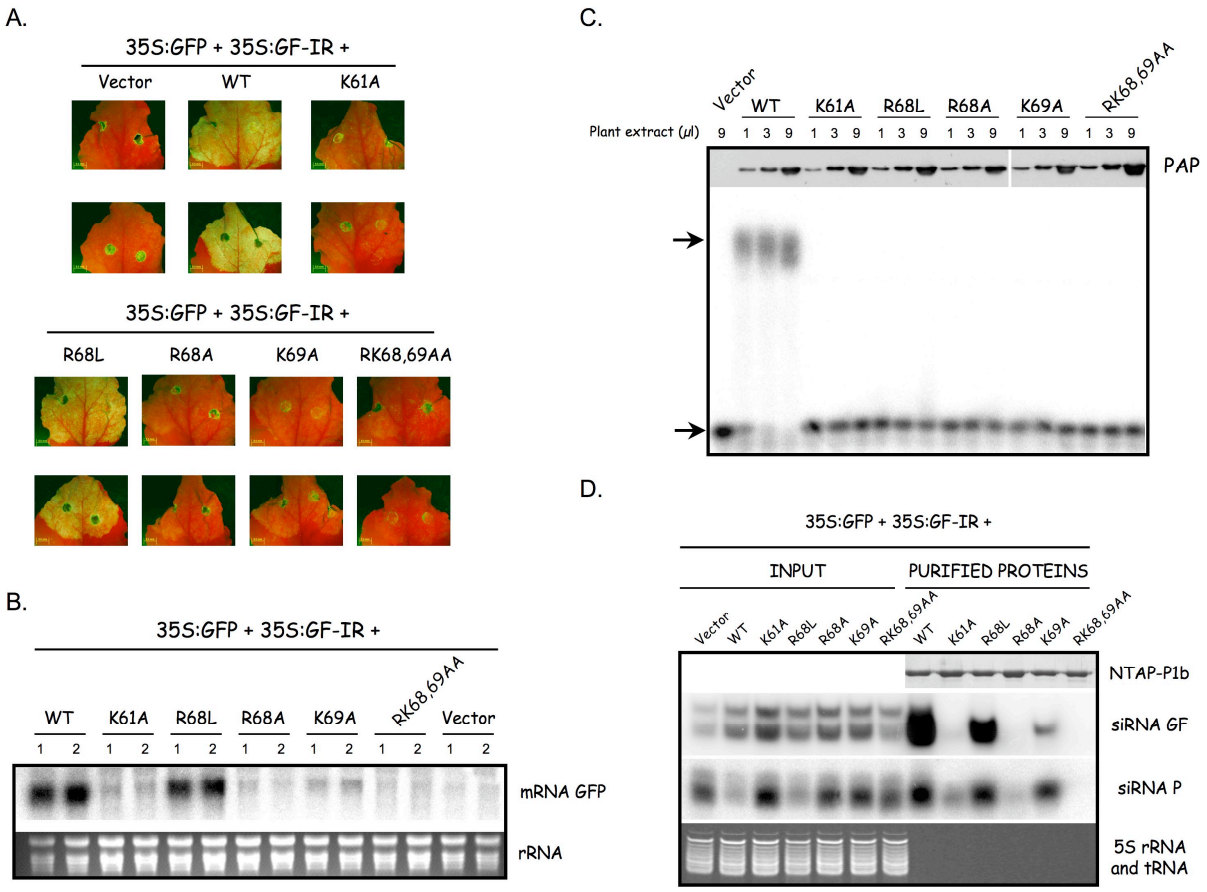


Figure R32. Silencing suppression activity and siRNA binding capacity of P1b mutant proteins. *N. benthamiana* plants were coinfiltrated with agrobacteria carrying p35S:GFP and p35S:GF-IR plus empty pBIN19 plasmid (Vector), wild type p35S-NTAP-P1b or derivatives of this plasmid with the indicated mutations. The infiltrated leaves were harvested at 4 dpi. (A) GFP fluorescence pictures taken under a fluorescence stereomicroscope. (B) Northern blot analysis of GFP mRNA extracted from infiltrated leaves. Agarose gel stained with BrEt is shown as loading control (rRNA). (C) siRNA binding analysis by EMSA. Crude protein extracts of infiltrated leaves (1, 3 or 9 µl) were incubated with ^{32}P-labeled 21-nt ds-siRNAs. Complexes were resolved by polyacrilamide gel electrophoresis and revealed by autoradiography. The amount of NTAP-P1b proteins present in each extract was estimated by Western blot analysis with PAP complex (on top of the panel). Arrows indicate bound and free ^{32}P-labeled 21-nt ds-siRNAs. (D) Northern blot analysis of both GF- and P-derived siRNAs from either co-infiltrated leaves (Inputs) or NTAP-P1b proteins purified by affinity chromatography (Purified Proteins). 5S rRNA and tRNA stained with BrEt are shown as loading control. The samples of purified NTAP-P1b proteins used for the siRNA extraction were subjected to SDS-polyacrylamide gel electrophoresis to assess their protein amounts (NTAP-P1b).

The precise correlation between the ability to bind siRNAs *in vivo* and the silencing suppression activity of the P1b mutants analyzed here strongly suggest that interaction with siRNAs plays a key role in the anti-silencing effect mediated by this RSS, and that the LxKA motif forms part of the siRNA binding domain of the protein.

III.5 Relevance of P1b and its RNA silencing suppression activity in the context of viral infections

Most of the monopartite members of family *Potyviridae* make use of HCPro to counteract the plant defense mechanisms mediated by silencing; however, this seems to be not the case for viruses from the *Tritimovirus* and *Ipomovirus* genera, which either lack the coding sequence of this protein (Janssen *et al.*, 2005; Li *et al.*, 2008; Mbanzibwa *et al.*, 2009) or express an HCPro protein without silencing suppression capacity (Giner *et al.*, 2010; Stenger *et al.*, 2007). Recent results have demonstrated that in these viruses a P1 serine-protease would be responsible for the anti-silencing activity (Giner *et al.*, 2010; Mbanzibwa *et al.*, 2009; Stenger, 2007), as it is the case of P1b, the strong RNA silencing suppressor of CVYV.

To assess the importance of CVYV P1b in the context of a viral infection, we have investigated the relevance of the RNA silencing suppression activity of this protein on virus pathogenicity and survival. Given the unavailability of an infectious cDNA clone for CVYV, two different approaches based on other viruses have been carried out; hence, a heterologous system based on the potexvirus PVX, and a homologous system based on the potyvirus PPV, were developed. Here, we show that P1b was able not only to enhance the symptoms and accumulation of PVX, but also to functionally replace HCPro in a potyviral infection. Interestingly, these P1b capacities correlated with its silencing suppression and siRNA binding activities.

III.5.1 Expression of CVYV P1b enhances PVX pathogenicity.

In order to assess the relevance of the strong silencing suppression activity of CVYV P1b in the course of a virus infection, sequences coding for wild type o mutated P1b were cloned in a PVX-derived vector (Fig. R33A). Both *N. benthamiana* and *N. clevelandii* plants were inoculated with the PVX-derived viruses by agroinfiltration. Systemic symptoms were detected at 5 days post-inoculation (dpi) in plants infected with empty PVX or with PVX expressing the CVYV P1b proteins. However, whereas plants infected with empty PVX and PVX expressing either P1b RK68,69AA or C89A, which lack silencing suppression activity, developed relatively mild symptoms, plants infected with PVX carrying wild type P1b suffered a generalized necrosis, and they died at 15-21 dpi (Fig. R33B). Although the extreme necrotic damage of leaves infected with PVX expressing wild type P1b precluded a precise quantitative assessment, Western blot analysis revealed higher accumulation of PVX CP in

plants infected with PVX-P1b than in those infected with empty PVX, PVX-P1b-RK68,69AA or PVX-P1b-C89A (Fig. R33C). Moreover, the Western blot analysis also showed the presence of P1b in plants infected with PVX-P1b-RK68,69AA and PVX-P1b-C89A, but again the accumulation levels were lower than in plants infected with PVX-P1b (Fig. R33C). Together, these results show that P1b, like other viral silencing suppressors (Pruss *et al.*, 1997), exacerbates PVX infection, and this pathogenicity enhancement is likely the result of a counterdefense activity of P1b, which correlates with silencing suppression activity.

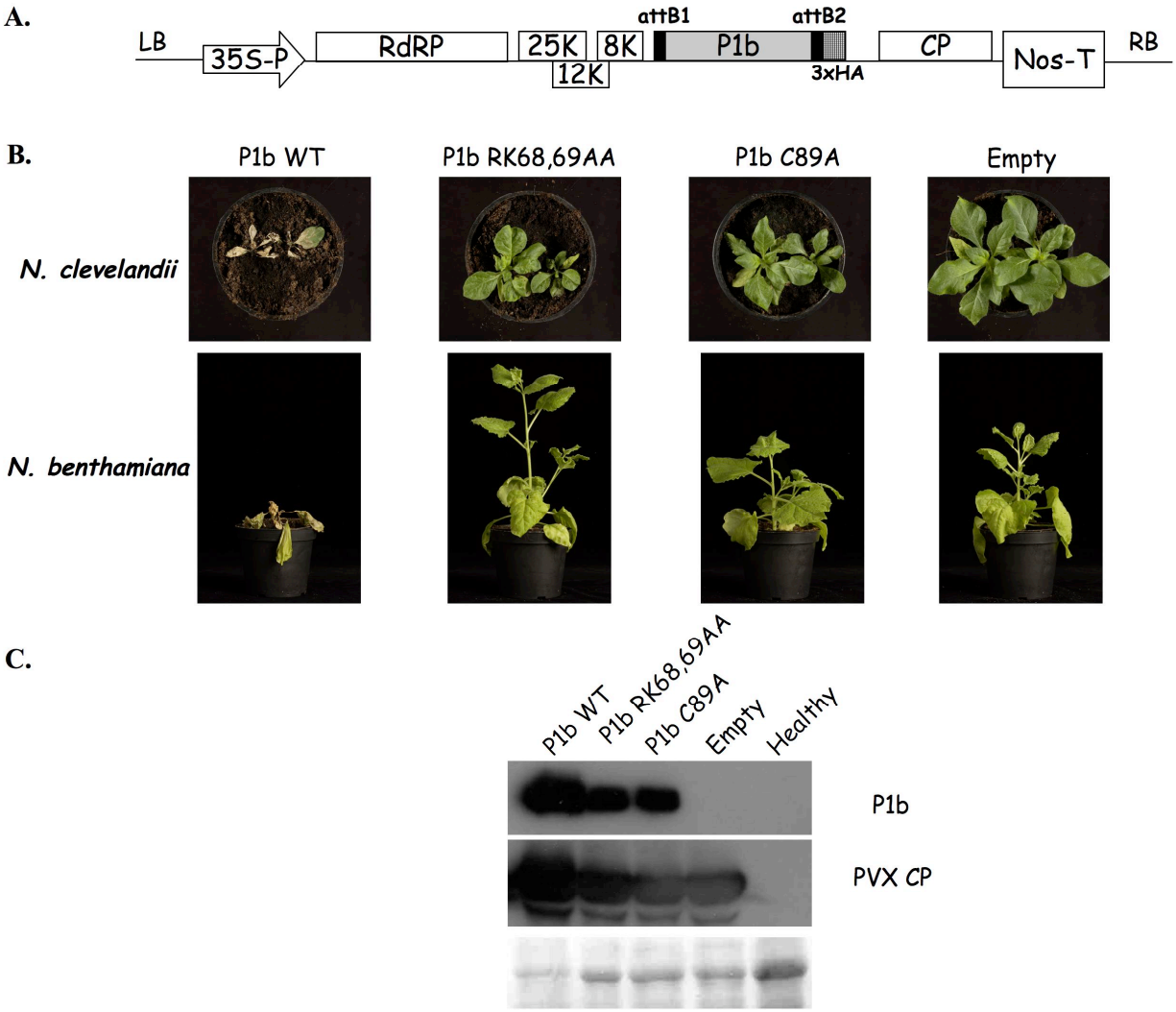


Figure R33. CVYV P1b enhances PVX pathogenicity. (A) Schematic representation of the GATEWAY-adapted T-DNA of pGWC-PVX with the CVYV P1b coding sequence inserted in its cloning site. (B) Symptoms of wild type PVX (empty) or PVX expressing CVYV P1b (wild type or the indicated mutants) in *N. clevelandii* and *N. benthamiana* plants. Photographs were taken at 21 dpi. (C) Western blot analysis with anti-CVYV P1b and anti-PVX CP sera of extracts of *N. benthamiana* leaves systemically infected with wild type PVX (empty) or PVX expressing CVYV P1b (wild type or the indicated mutants). Healthy *N. benthamiana* leaves were also analyzed. The leaves were harvested at 10 dpi. The blot stained with Ponceau red is shown at the bottom as a loading control.

III.5.2 CVYV P1b can functionally replace HCPro in a PPV infection

To test the ability of CVYV P1b to support PPV infection, the HCPro coding sequence was replaced by that of CVYV P1b in the infectious cDNA clone pICPPV-NK-GFP, which also expresses the GFP to monitor the viral infection *in planta* (Fernández-Fernández *et al.*, 2001). For simplicity these clones are named here according the N-terminal regions of their polyproteins (P1HC, P1Pb, Fig. R34)

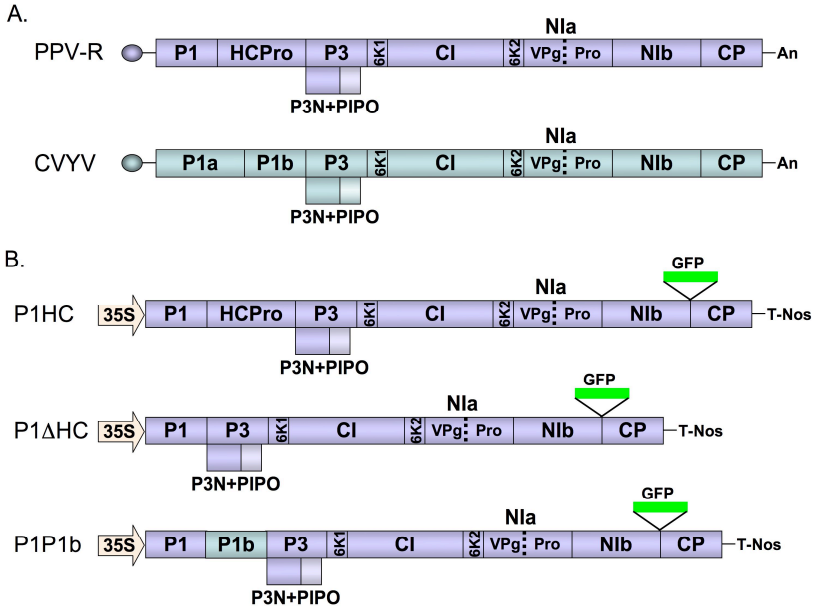


Figure R34. Schematic representation of PPV/CVYV-derived constructs used in the infection assays. (A) Genome maps of PPV and CVYV. (B) cDNAs derived from pIC-PPV-NK-GFP. The coding sequence of the GFP protein inserted between the NIb and CP cistrons is represented with a green rectangle.

N. benthamiana and *N. clevelandii*, were biolistically inoculated with the different cDNA clones, and the infection processes were tracked by monitoring of the inoculated plants under visible and UV lights. The chimeric PPV expressing P1b showed in both kinds of plants a high infectivity (usually 100%), similar to that of wild type PPV expressing HCPro. However, there was a small delay of 1-2 days in the appearance of symptoms and GFP fluorescence in upper noninoculated leaves of plants infected with the P1b-expressing virus with respect to plants infected with wild type PPV. Both P1P1b and P1HC caused systemic chlorotic mottling in *N. clevelandii* and *N. bethamiana*, but these symptoms were more severe in the plants infected with the P1b-expressing virus. In addition, *N. bethamiana* plants infected with P1Pb showed striking leaf distortion and edge curling, but they were notably less stunted than those infected with wild type PPV (P1HC) (Fig. R35A and B, top panels). Although visible symptoms were more prominent in leaves of P1P1b-infected plants, the virus-derived GFP fluorescence was less intense in these plants than in those infected with P1HC. Western blots analysis of leaf extracts prepared at 21 dpi showed that high levels of CP accumulated in *N. clevelandii* and *N. benthamiana* plants inoculated with P1P1b, but

some lower than those detected in extracts from plants inoculated with P1HC (Fig. R35A and B, bottom panels). Immunoreactions with anti-P1b and anti-HCPro specific antibodies confirmed that each virus expressed the expected RSS (Fig. R35A and B bottom panel). These results indicate that CVYV P1b can functionally replace HCPro in a PPV infection.

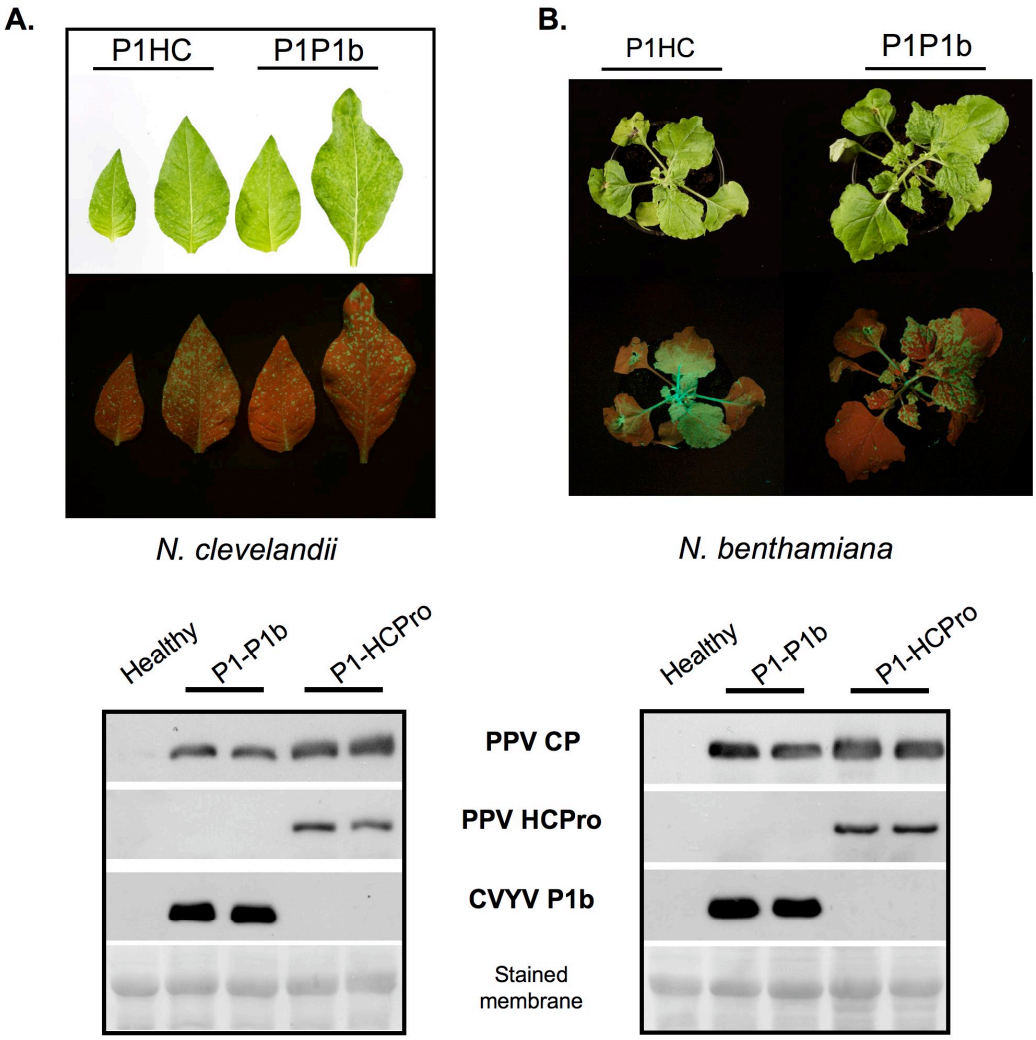


Figure R35. A chimerical PPV in which HCPro has been replaced by CVYV P1b (P1P1b) infects *N. clevelandii* and *N. benthamiana* plants. Top panels: Symptoms and GFP expression of plants infected by the chimera P1P1b and wild type PPV (P1HC). Pictures of detached *N. clevelandii* upper noninoculated leaves (A) and whole *N. benthamiana* plants (B) taken under visible light (upper row) and UV irradiation (lower row) at 21 dpi. Bottom panels: Western blots of systemically infected leaves from two plants collected at 21 dpi. Polyclonal sera specific for CP and HC-Pro of PPV, and CVYV P1b were used for the immunodetections. The stained membrane is shown as loading control.

III.5.3 The silencing suppression activity of P1b is needed for its ability to replace HCPro in the PPV infection

In order to assess the relevance of P1b-mediated silencing suppression in the context of a potyviral infection, we constructed two variants of the P1P1b full-length cDNA coding for P1b proteins with mutations that abolish its RNA silencing activity: RK68,69AA in the LxKA motif and C89A in the putative Zn finger (Figs. R18 and R19). The C93A mutation, which

does not affect the P1b silencing suppression activity, was engineered in a control PPV P1P1b clone. In addition, a PPV cDNA clone lacking HCPro was also generated (P1ΔHC, Fig. R34). *N. benthamiana* plants were inoculated with DNA of these PPV clones by microprojectile bombardment, and the infection process was followed by monitoring of GFP fluorescence and symptom expression. Green fluorescence in inoculated and upper non-inoculated leaves, and systemic symptoms were observed in plants inoculated with clones coding for an active RNA silencing suppressor (P1HC, P1P1b and P1P1b C93A), but not in those inoculated with P1P1b RK68,69AA or P1P1b C89A, which code for inactive P1b mutants, or P1ΔHC, which does not encode a known silencing suppressor (Fig. R36A).

A.

Construct	Suppressor protein	Suppressor Activity	Symptoms and GFP (%)
P1HC	HC-Pro	+	100
P1ΔHC	-	-	0
P1P1b	P1b	+	100
P1P1b RK68,69AA	P1b RK68,69AA	-	0
P1P1b C89A	P1b C89A	-	0
P1P1b C93A	P1b C93A	+	100

B.

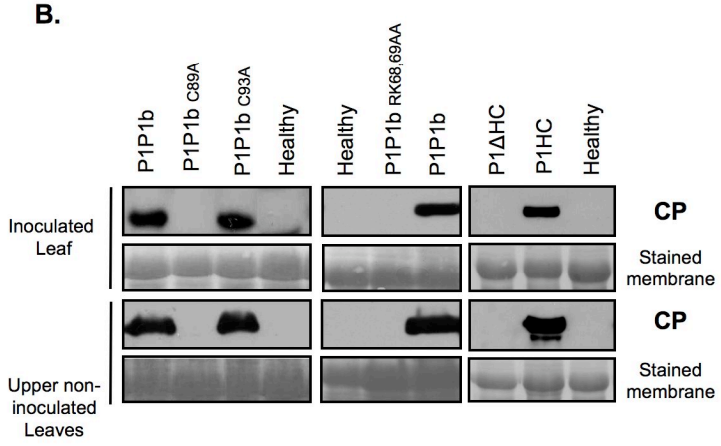


Figure R36. PPV lacking an active RSS is not able to infect *N. benthamiana*. (A) Result of visual inspection of *N. benthamiana* plants inoculated with PPV cDNA clones differing in their encoded RSS (B) Western blots of protein extracts from pools of either inoculated (7 dpi) or upper non-inoculated 21 dpi) leaves of four *N. benthamiana* plants inoculated with the indicated cDNA clones. A polyclonal serum specific for PPV CP was used for the immunodetection. The stained membrane is shown as loading control.

Western blot analyses of protein extracts from both inoculated and upper leaves confirmed the visual observations, and showed no viral CP accumulation in plants inoculated with P1P1b RK68,69AA, P1P1b C89A or P1ΔHC, while large amounts of viral CP were detected in the plants inoculated with clones coding for active RSSs (P1HC, P1P1b and P1P1bC93A) (Fig. R36B). These results strongly suggest that the RNA silencing suppression activity, mediated by either HCPro or P1b, is essential to maintain a PPV infection.

Discussion

IV. DISCUSSION

IV.1 The P1 and HCPro proteins in the evolution of the family *Potyviridae*

Our understanding of plant virus evolution is improving as consequence of renewed interest in the subject, caused in part because plant viruses serve as excellent model systems (reviewed in García-Arenal *et al.*, 2003; Gibbs & Ohshima, 2010; Gibbs *et al.*, 2008; Roossinck, 2003; Roossinck, 2005). Some evidence of virus coevolution between hosts and their vectors is available, providing information on virus origin and evolutionary history (Lovisolo *et al.*, 2003; Pagan *et al.*, 2010). Regarding the *Potyviridae* family, data reported in chapter III.1 illustrates the extensive evolutionary divergence of the P1 region in *Potyviridae* family members. Recent information has come out supporting the existence of two kinds of P1s (here termed as classical and P1b-like). Thus, the specific features for P1b-like P1s described in the chapter III.1.3 are shared for P1 proteins of other ipomoviruses and tritimoviruses whose genomic sequence has been recently reported: the P1b protein from the ipomovirus SqVYV, and the single P1s from the ipomovirus CBSV and the tritimovirus WEqMV. Interestingly, genomic sequence information of P1s from the still unclassified TriMV, SCSMV and BLVY indicate that they share some features of the P1b-like group, such as conservation of the LxKA motif (Fig. 30) or low pI. The conclusive evidence for the occurrence of functional diversification between classical and P1b-like P1s was reinforced by results showing that, besides CVYV P1b, the P1s from the tritimovirus WSMV, and from the ipomoviruses SPMMV and CBSV, have RNA silencing suppression activity, in spite of the fact that these three viruses produce HCPro proteins (Giner *et al.*, 2010; Mbanzibwa *et al.*, 2009; Stenger, 2007).

All this information, allow us to envisage different models for the evolution of the P1-HCPro region in the family *Potyviridae* (Fig. D1). In an intuitive scenario, the ancestor of the *Potyvirus* genus would have a single P1 gene (Fig. D1A). Duplication of potyvirus gene coding for a P1 protein with a Zn finger-like motif at its N-terminus would have produced the ancestor of the P1s of ipomoviruses and tritimoviruses, as well as those of the unclassified TriMV, SCSMV and BLVY. Each of the P1 copies would have evolved independently and adopted different functions. In the evolutionary branch containing the ipomovirus SPMMV, tritimoviruses, TriMV, SCSMV and BLVY, the function of the first protease domain would have been superfluous and either partially or totally eliminated. In the branch containing the ipomoviruses CVYV, SqVYV and CBSV, the functions assumed by the two P1s of CVYV and SqVYV would have allowed the viruses to dispense with the HCPro gene, whereas the

functions assumed then by the only P1b-like protein make the P1a dispensable in some cases. However, since the first protease domain of CVYV and SqVYV more closely resembles the single protease domain of potyviruses than that of P1b-like P1s, P1 duplication and divergence of the two resulting P1 copies could have taken place before the evolutionary pathways of potyviruses, ipomoviruses, tritimoviruses and other related potyvirids split. According to this hypothesis, potyviruses would have derived from a deletion of the second P1 protease domain (Fig. D1B). Importantly, gene duplication might have facilitated the functional diversification of P1, although during the course of evolution some of these functions may have become dispensable in some lineages. It is interesting to remark that present potyvirid have different combinations of proteases at the N-terminus of their polyprotein but not more than two. This fact questions how the ancestor containing two P1 plus one HCPro, which is postulated in the two models raised in Fig. D1, could have been positively selected. In any case, the models presented in Fig. D1 are quite and further research will be required to unravel the details of the evolution of the *Potyviridae* family.

The *in silico* sequence analysis reported in the chapter III.1 shows extensive divergence even among the classical potyviral P1s, and provides clear evidence that RNA recombination has played a crucial role in this diversification. These data indicate that frequent recombination events at the P1 gene might take place even between viruses of different genera in the *Potyviridae* family (section III.1.5). RNA recombination appears to have played an important role in host adaptation (Figs. R7 and R10). Most recombination events described here affect P1 sequences upstream of the protease domain, suggesting that the N-terminal part of the protein could be the region involved in interactions with plant factors contributing to host adaptation. Recent experimental data also support a role for P1 in pathogenicity and host range definition of potyviruses (Chiang *et al.*, 2007; Salvador *et al.*, 2008). In addition, unpublished data from our laboratory show that the expression of CVYV P1b instead of HCPro could facilitate the replication of PPV in *Cucumis sativus*, the natural host of CVYV (A. Carbonell, G. Dujovny, J.A. García and A. Valli, manuscript in preparation) which would indicate that P1b-like P1s could also contribute to host specification. Unravelling the molecular interactions of P1 proteins with host factors will be the next step to shed light on our understanding of the roles of this proteins in viral pathogenicity and of the constraints and driving forces of potyviral evolution related with them.

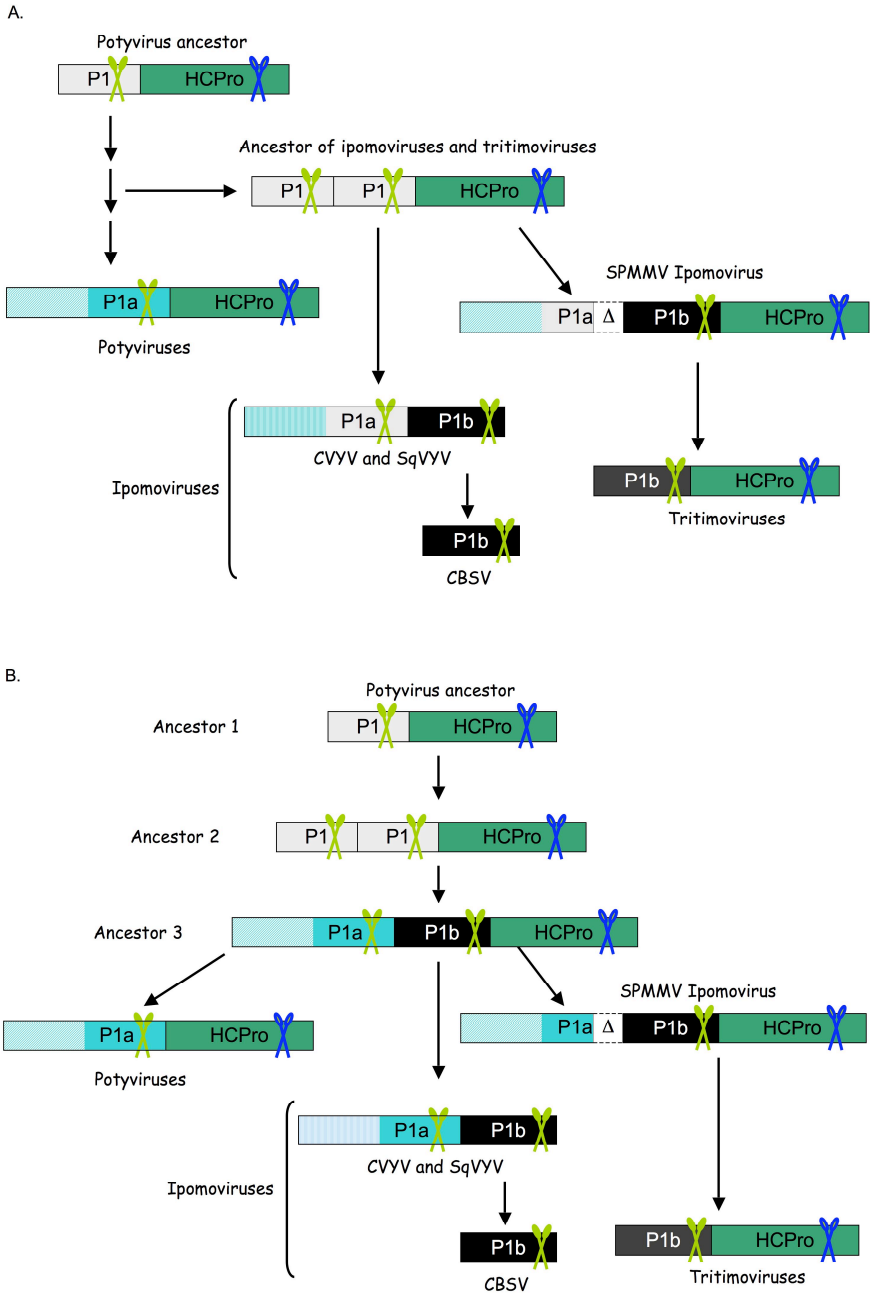


Figure D1. Panels A and B show two alternative models for evolution of the P1-HCPro region in the *Potyviridae* family. The conserved protease domains and the divergent P1 N-terminal domains are represented by solid and framed filling, respectively. Green and blue scissors mark serine protease and cysteine protease domains, respectively. Different colours illustrate different evolutionary lineages.

IV.2 Two P1 serine proteases are placed at the N terminus of the CVYV polyprotein, P1a and the peculiar P1b

An *in silico* analysis identified two homologous serine protease domains at the N-terminal region of the CVYV polyprotein, suggesting that the initially proposed long P1 protein was formed of two independent proteins P1a and P1b (Fig. R1). Transient *in planta* expression of the complete P1 region by agroinfiltration revealed the predicted proteolytic activity of P1a (Fig. R11). A similar approach verified the proteolytic activity of P1b, which

was abolished by mutations at H221 and S264 (Fig. R16 and R17), confirming the *in silico* identification of these residues as components of the catalytic active site of the protein. Cleavages splitting P1a from P1b (Fig. R11), and P1b from downstream sequences (Fig. R17B) appeared to be very efficient in the agroinfiltration system, and also in CVYV-infected cucumber plants (Fig. R17C). Thus, although CVYV lacks a cysteine proteinase HCPro, its strategy of genome expression is quite similar to that of most of the viruses of the family *Potyviridae*, involving polyprotein processing by three virus-encoded proteinases. Further research will be required to ascertain specific details of CVYV polyprotein maturation and the possible functional relevance of P1a-P1b and other partially processed products.

IV.3 CVYV P1b, a novel silencing suppressor into *Potyviridae* family

Although alternative strategies for escaping RNA silencing have been proposed (Liu *et al.*, 2005; Schwartz *et al.*, 2002; Taliansky *et al.*, 2003), most plant RNA viruses appear to depend on virus-encoded suppressor proteins to counteract this antiviral defense mechanism (Burgyán, 2008; Diaz-Pendon & Ding, 2008). In some cases, the virus has more than one silencing suppressor (Cañizares *et al.*, 2008; Lu *et al.*, 2004). Until recently, HCPro was thought to be the only RSS in the family *Potyviridae* (Anandalakshmi *et al.*, 1998; Brigneti *et al.*, 1998; Kasschau & Carrington, 1998), although the preceding protein, P1, had been suggested to enhance the silencing suppression activity of HCPro (Anandalakshmi *et al.*, 1998; Kasschau & Carrington, 1998; Pruss *et al.*, 1997; Rajamäki *et al.*, 2005). All monopartite members of the family *Potyviridae* characterized until now concur in having an HCPro-like cysteine protease at the second position of the viral polyprotein, except for the ipomoviruses CVYV, SqVYV and CBSV (Janssen *et al.*, 2005; Li *et al.*, 2008; Mbanzibwa *et al.*, 2009), which lack the HCPro cistron. This raises the question about how these viruses can supply the multiple HCPro activities, especially those needed to suppress silencing. The results shown in the chapter III.2 demonstrate that the second P1 copy of CVYV, P1b, is able to suppress silencing in different experimental systems (Figs. R13, R14 and R15). Also the single P1 of CBSV, another ipomovirus lacking HCPro, has been reported to have RNA silencing suppression activity (Mbanzibwa *et al.*, 2009). However, this activity is not restricted to P1 proteins of viruses defective in HCPro. It has been also demonstrated for the P1 proteins of the ipomovirus SPMMV (Giner *et al.*, 2010) and the tritimovirus WSMV (Stenger, 2007), and it is likely that RNA silencing suppression activity is a general feature of P1b-like P1s.

The most compelling sequence similarity among P1b-like P1s is at its C-terminal, which includes a typical serine protease signature (Fig. R16). However, the protease activity of CVYV P1b is not essential for its RNA silencing suppression activity (Figs. R18 and R19). Also the potyviral HCPro does not depend on its cystein proteinase activity to suppress silencing (Kasschau & Carrington, 2001). In spite of this fact, the proteolytic processing activity of HCPro is essential for the potyviral infection (Kasschau & Carrington, 1995). It would be interesting to unravel which potyviral process, likely unrelated to silencing suppression, the protease activity of HCPro is required for, and to determine whether a similar requirement for the serine proteinase activity of P1b affects CVYV infection.

The N-terminal region of P1b-like P1s is barely conserved, but it is possible to recognize two partially conserved motifs. No matches have been found in protein domain databases for the first motif, which is in a basic region and has a LxKA signature (Figs. R16 and R30). The second one is characterized by several conserved cysteines, which are arranged as zinc fingers of the type $Cx_2Cx_nCx_2C$ (Mackay & Crossley, 1998). The fact that point mutations in the LxKA motif and in the putative zinc finger abolish the RNA silencing suppression activity of CVYV P1b (Figs. R18, R19 and R32) highlights the involvement of the N-terminal domain of P1b in suppression of silencing and hints the functional relevance of these conserved motifs.

Both BiFC *in planta* (Fig. R21), and gel filtration FPLC *in vitro* (Fig. R22) show that, as other RSSs (Chao *et al.*, 2005; Chen *et al.*, 2008; Lingel *et al.*, 2005; Plisson *et al.*, 2003; Ruiz-Ferrer *et al.*, 2005; Vargason *et al.*, 2003; Ye *et al.*, 2003; Ye & Patel, 2005), CVYV P1b is able to interact with itself. P1b mutational analysis suggested that efficient P1b self-interaction requires preservation of the zinc finger domain. Thus, P1b proteins with mutations affecting the cysteines predicted to form the zinc finger displayed a very weak interaction in the BiFC assay (Fig. R21), which correlated with a nonspecific aggregation pattern observed by FPLC in the cases of C103A and C106A mutants (Fig. R22). The fact that the C89A mutant usually showed an FPLC elution profile similar to that of the wild type protein (Fig. R22) suggests that the deleterious effect of the C89A point mutation is less severe than that of the C103A and C106A mutations, and, in consequence, was only clearly detected *in planta*. A possible explanation for this apparently milder effect of the C89A mutation is that H90 or C93, which are located very close to C89 (Fig. R16), could partially substitute for this residue in the zinc finger configuration. Zinc fingers were initially identified as protein motifs involved in nucleic acid recognition (Klug & Rhodes, 1987), but it is now known that they are also involved in protein-protein interactions (Cox & McLendon, 2000; Gamsjaeger *et al.*,

2007). Our results suggest that a zinc finger formed by C86, C89, C103 and C106 is involved in dimerization of CVYV P1b and that disturbance of this interaction causes nonspecific aggregation and inactivation of the protein. A similar overall destabilization of the protein structure caused by disruption of zinc finger-mediated self-interactions has been described previously (Payre *et al.*, 1997). Interestingly, zinc finger motifs are present in a number of silencing suppressors of plant viruses, including the potyviral HCPro, (for instance Bragg & Jackson, 2004; Chiba *et al.*, 2006; Mérai *et al.*, 2006; Rakitina *et al.*, 2006; Urcuqui-Inchima *et al.*, 1999; van Wezel *et al.*, 2002; Zhou *et al.*, 2006) and in a subset of P1a-like P1 proteins (Fig. R9), but the specific relevance of these motifs is still unclear. In contrast with the zinc finger motif, the LxKA motif appeared not to be involved in P1b self-interaction (Figs. R21 and R22), suggesting that it should participate in other functions required for the RNA silencing suppression activity (see below).

IV.4 Unravelling the RNA silencing suppression mechanism of CVYV P1b, and its consequences

Viral silencing suppressors largely differ in amino acid sequence, and the data available suggest that they also have quite different mechanisms of action. CVYV P1b and PPV HCPro show a complete sequence disparity, however, these RSSs behaved in a very similar manner in all the experimental systems that we used. P1b and HCPro are able to suppress not only the RNA silencing induced by sense RNA (Fig. R13) but also that triggered by an inverted repeated RNA (Fig. R14) indicating that both RSSs act on a silencing step downstream dsRNA formation. Moreover, the fact that expression of P1b and HCPro does not prevent siRNA accumulation (Figs. R13, R14, R18 and R19) indicates that these RSSs neither disturbs the synthesis of siRNAs nor enhance their degradation. HCPro has been shown to interact with siRNAs (Lakatos *et al.*, 2006), and it has been suggested that this interaction could inactivate them, by interfering with their loading in effector complexes (Lakatos *et al.*, 2006) and with their contribution to the silencing amplification step (Mlotshwa *et al.*, 2008a; Moissiard *et al.*, 2007; Zhang *et al.*, 2008). The ability of CVYV P1b to interact with siRNAs both *in vitro* and *in vivo* (Figs. R23 and R28) suggests that these RSSs could also suppress silencing by a similar siRNA sequestration mechanism. In agreement with this hypothesis, whereas the silencing suppression activity of CVYV P1b is not associated in the agroinfiltration assay with a notable change in the levels of total siRNA accumulation, the

amount of secondary RNAs was much lower in leaves expressing active P1b than in those expressing inactive P1b variants (Fig. R32).

The weak and variable interference effect of HCPro and CVYV P1b on RNA silencing spread in GFP transgenic *N. benthamiana* 16c plants (Fig. R15) is also compatible with this proposed mechanism. The signals involved in the RNA silencing propagation appear to be small RNAs (Dunoyer *et al.*, 2005; Dunoyer *et al.*, 2010; Himber *et al.*, 2003; Molnar *et al.*, 2010). P1b and HCPro could have a dual effect on these molecules. On one hand, their local silencing suppression activity would facilitate a very active expression of the GFP trangene, which would be the substrate to produce larger amounts of siRNAs involved in silencing spreading. On the other hand, HCPro and P1b would bind to these siRNAs partially preventing their activity. In this scenario, the actual balance of the two effects and the specific requirement of the silencing signal would determine the efficiency of the short and long distance silencing spread.

Binding to dsRNA is a usual strategy used by RSSs to suppress silencing. A number of RSSs bind dsRNAs without size-specificity, and are thought to protect small RNA precursors from cleavage by Dicer-like enzymes (Mérai *et al.*, 2006). Other RSSs, as potyviral HCPro and tombusviral P19, show strong size-preference for small RNAs of 21 nt, and act by preventing small RNA loading in RISC (Lakatos *et al.*, 2006). Moreover, it has been suggested that some RSSs, as the protein B2 of the nodavirus *Flock house virus* could use both strategies to achieve a more efficient suppression (Chao *et al.*, 2005). By *in vitro* EMSA analyses we showed that whereas CVYV P1b interacts with dsRNA molecules (Figs. 23 and 25A), it is unable to bind either ss-RNA or ss- or ds-DNA (Fig. R25A and B). Moreover, EMSA competition tests indicated that P1b has a preference for binding to 21-nt siRNAs when compared to 24-nt or 26-nt siRNAs (Fig. R26), as it has been previously reported for HCPro and P19 (Lakatos *et al.*, 2006). However, in spite of sharing the same size-preference, the RNA binding of these three RSSs appears to have important specific features. It has been reported that the siRNA binding of the P19 protein of the tombusvirus CIRV depends on 5' terminal phosphate, but does not require the 3' 2-nt overhangs of natural siRNAs (Vargason *et al.*, 2003). In contrast, the TEV HCPro was shown to have higher binding affinity for siRNA with 2-nt overhangs than for blunt-ended duplexes (Lakatos *et al.*, 2006), and this result was confirmed here for PPV HCPro (Fig. R27B). Our data show that siRNA binding of CVYV P1b differs from that of either P19 or HCPro. CVYV P1b is similar to P19 and differs from HCPro in the ability to bind blunt-ended siRNA duplexes at least as efficiently as siRNAs with 2-nt overhangs (Fig. R27A). On the other hand, CVYV P1b differs from both

P19 (Vargason *et al.*, 2003) and HCPro (Fig. R27D) in its ability to bind with similar affinity siRNAs with a phosphate or a free OH at their 5' ends (Fig. R27C). These different behaviours, together with the lack of detectable sequence similarity between the three RSSs, highlight how very similar functional strategies, specific binding of 21-nt RNA duplexes, can be attained by independent evolutive pathways.

The purification by affinity chromatography of CVYV P1b expressed in *N. benthamiana* by agroinfiltration demonstrated the ability of this RSS to bind small RNAs *in vivo*. Northern blot (Fig.R28B) and deep sequencing analysis of these small RNAs (Fig. R29) showed a size-preference for molecules of 21-22 nt, confirming the *in vitro* EMSA results. The deep sequencing analysis also showed that not only siRNAs derived from the agroinfiltrated plasmids, but also plant endogenous small RNAs (Fig. R29B), including known miRNAs (Table D1), were bound to P1b.

Table D1: List of known miRNA molecules present in different *Nicotiana benthamiana* samples sequenced by Solexa-Illumina technology.

miRNA	Homologous[1]	Mut[2]	Sequence (5'-3')	Length	Nb[3]	Nb + P1b[3]	CoP-P1b[3]
miRNA156	ath-miRNA156a	-	TGACAGAAGAGAGTGAGCAC	20	1916,8	3952,7	2180,6
miRNA156	bna-miRNA156a	-	TGACAGAAGAGAGTGAGCACA	21	2,2	143,1	124,4
miRNA157	ath-miRNA157a	-	TTGACAGAAGATAGAGAGCAC	21	3973,6	6078,0	5292,9
miRNA157	ath-miRNA157d	-	TGACAGAAGATAGAGAGCAC	20	218,6	112,1	112,1
miRNA159	ath-miRNA159a	-	TTTGGATTGAAGGGAGCTCTA	21	60,1	99,0	279,2
miRNA159*			GAGCTCCTTGAAGTCCAACAG	21	-	2,7	13,4
miRNA162	ath-miRNA162a	-	TCGATAAACCTCTGCATCCAG	21	-	2,1	1,1
miRNA162*			GGAGGCAGCGGTTTATCGATC	21	-	-	2,2
miRNA164	ath-miRNA164a	-	TGGAGAAGCAGGGCACGTGCA	21	174,3	31,6	114,4
miRNA164*			CATGTGCCTGTCTTCCCCATC	21	-	-	5,6
miRNA166	ath-miRNA166a	-	TCGGACCAGGCTTCATTCCCC	21	146,4	438,8	1935,1
miRNA166*			GGAATGTTGTCTGGCTCGAGG	21	4,2	36,6	134,7
miRNA167	ath-miRNA167a	-	TGAAGCTGCCAGCATGATCTA	21	8,7	14,4	72,9
miRNA168	ath-miRNA168a	1	TCGCTTGGTGCAGGTCGGGAC	21	13,7	26,1	20,2
miRNA168*			CCCGCCTTGCATCAACTGAAT	21	3,1	5,4	15,6
miRNA171	zma-miR171f	1	TTGAGCCGTGCCAATATCTCA	21	0,5	0,7	2,2
miRNA171*			AGATGTTGGTGCGGTTCAATG	21	-	3,4	12,2
miRNA172	ath-miRNA172a	-	AGAATCTTGATGATGCTGCAT	21	1,1	1,4	2,2
miRNA319	ath-miRNA319a	-	TTGGACTGAAGGGAGCTCCCT	21	1,1	0,7	2,2
miRNA390	ath-miRNA390a	-	AAGCTCAGGAGGGATAGCGCC	21	2,2	4,1	10,1
miRNA396	ath-miRNA396b	-	TTCCACAGCTTTCTTGAACTT	21	0,5	2,1	4,5
miRNA396*			GTTCAATAAAGCTGTGGGAAG	21	3,7	49,4	70,1
miRNA397	sly-miRNA397	1	ATTGAGTGCAGCGTTGATGAA	21	1,6	13,1	32,5
miRNA398	osa-miRNA398b	1	TATGTTCTCAGGTCGCCCCTG	21	1,6	2,1	6,7
miRNA398	ath-miRNA398a	-	TGTGTTCTCAGGTCACCCCTT	21	-	1,4	2,2
miRNA403	ath-miRNA403	-	TTAGATTCACGCACAAACTCG	21	-	3,4	10,1
miRNA403*			CGTTTGTGCGTGAATCTGACA	21	-	19	26,7
miRNA408	ppt-miRNA408b	-	TGCACTGCCTCTTCCCTGGCT	21	0,5	4,1	15,7
miRNA408*		-	GCAGGGACGAGGTAGAGCATG	21	13,6	3028,3	5175,4

[1]Identification of known miRNAs was done by miRProf and the searching for specific miRNA species was done with "search" tool on miRBASE (http://www.mirbase.org).
[2]Number of mutations respect to the homologous miRNA.
[3]Number of reads per 100000 sequences of *planta* small RNAs (see Fig. R29A).

The expression of P1b caused a drastic change in the size pattern of endogenous small RNAs of the plant (Fig. R29B). The major peak was at 24 nt in wild-type *N. benthamiana* plants, as it is usual in angiosperm plant species (Dolgosheina *et al.*, 2008), but shifted to 21 nt upon P1b expression. Although this enrichment in 21-nt small RNAs is probably the result of specific P1b binding and consequent stabilization, additional experiments are necessary to rule out possible effects of P1b in enhancing 21-nt small RNA synthesis or 24-nt small RNA degradation, or in repression of the accumulation of the 24-nt species. It has been shown that binding of different RSSs to small RNAs cause a broad range of positive and negative effects on their levels of accumulation and activity, resulting in drastic development disturbances in the plant (Chapman *et al.*, 2004; Dunoyer *et al.*, 2004; Kasschau *et al.*, 2003; Mallory *et al.*, 2002). Thus, the disturbances caused by P1b in the small RNA populations are expected to produce drastic effects in the physiology of the plant. In agreement with this assumption, *A. thaliana* plants transformed with a transgene expressing CVYV P1b have severe developmental defects that positively correlated with P1b accumulation levels (Fig. D2). Further research is required to associate definite effects of P1b on particular small RNAs with particular phenotypic alterations.

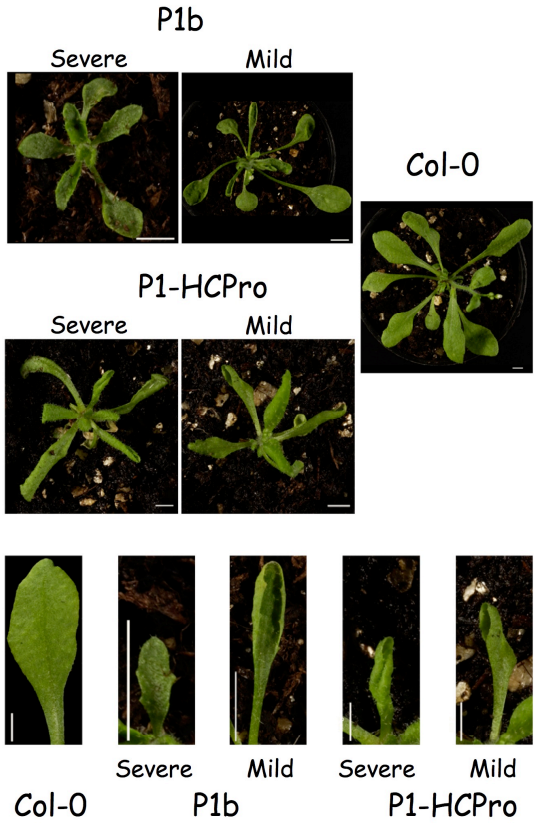


Figure D2. RSS expression can disturb plant development. Developmental defects induced in transgenic *A. thaliana* plants expressing the RSSs P1b from the ipomovirus CVYV and P1-HCPro from the potyvirus PPV. Bars, 5mm.

IV.5 Identification of a siRNA-binding domain in CVYV P1b

RNA binding is an activity largely widespread in proteins with very different functions, and a number of motifs related with this activity have been identified (Burd & Dreyfuss, 1994; Hall, 2002; Saunders & Barber, 2003). Computer analysis did not identify any canonical RNA binding motif in CVYV P1b, as is also the case for other viral RSSs with siRNA binding activity (Collins & Cheng, 2005). However, since the LxKA is rich in basic amino acids (Fig. R30), and these residues have been shown to contribute to the RNA binding of several RSSs (Bucher *et al.*, 2004; Chao *et al.*, 2005; Fenner *et al.*, 2007; Haasnoot *et al.*, 2007; Lingel *et al.*, 2005; van Rij *et al.*, 2006; Vargason *et al.*, 2003; Ye *et al.*, 2003) this conserved domain is a good candidate to be involved in the interaction of CVYV P1b with siRNAs. The results described in chapters III.3 and III.4 show that mutations to alanine of K61, R68, K69, or both R68 and K69 cause a drastic disturbance in the RNA silencing suppression activity of P1b, and abolish its ability to bind siRNAs *in vitro* (Figs. R24 and R32A-C). There are some reasons suggesting that the effect of these mutations is not caused by a global effect in the net charge of the protein. First, whereas typical P1 proteins of potyviruses are invariably highly basic, CVYV P1b has an estimated pI of 5.1, similar to that of the rest of P1b-like proteins (Fig. R1). Moreover, the double mutation KR10,11AA, which removes two positive charges in a non-conserved basic domain at the N-terminus of the protein did not affect siRNA binding and silencing suppression activity (Fig. R31). Also pointing to structural features of the LxKA domain, rather than solely positive charge, as involved in siRNA binding, we found that the functional effects of replacing R68 by alanine or by leucine, the residue present at this position in the P1 of the ipomovirus type member, SPMMV, were quite different. Both mutations prevented siRNA binding in the *in vitro* EMSA (Fig. R32C), however, in contrast with the drastic effect of R68A on RNA silencing suppression activity, R68L suppressed silencing with high efficiency (Fig. R32A and B). This apparent contradiction was untangled by a pull down assay revealing the small RNAs that are bound *in vivo* by the different mutant proteins. Whereas no (RK68,69AA), or very little amount of (K61A, R68A and K69A), siRNAs were pulled down by the silencing suppression-deficient mutants, the R68L protein bound small RNAs in the plant with notable efficiency, although not as high as that of wild type P1b (Fig. R32D). Interestingly, the K69A mutant, which appeared to protect mRNA from degradation above the background levels (Fig. R32B), also retained some more small RNAs than the K61A and R68A mutants (Fig. R32D). An important inference of all these results is that whereas *in vitro* EMSA is very useful to analyze

specific biochemical features of the siRNA-protein interactions, many functional aspects of the interaction only can be unravelled by studies under *in vivo* conditions.

All these results suggest an specific involvement of the LxKA domain for siRNA binding of CVYV P1b. In addition, the precise correlation between *in vivo* small RNA binding and silencing suppression activity of P1b proteins with mutations at this region strongly supports the conclusion that CVYV P1b blocks silencing by interfering with a siRNA function, which was discussed in the section IV.4. How this interference takes place is still unknown.

IV.6 Anti-silencing activity of CVYV P1b in the context of a viral infection

Most of the data reported here have been obtained testing activities of CVYV P1b out of a viral context. However, although the unavailability of an infectious CVYV cDNA clone has precluded a direct reverse genetics analysis of the role of P1b in CVYV infection, we have been able to develop two viral systems to assay the contribution of this protein and its capacity to counteract RNA silencing in viral infections. In the first approach we made use of a heterologous system based on PVX-derived recombinant viruses, which has been previously employed to study the ability of RSSs to stimulate virus infection (Pruss *et al.*, 1997). We observed that CVYV P1b, like potyviral HCPro and other RSSs, drastically enhances PVX symptoms, and this enhancement appears to be the result of a more efficient virus infection (Fig. R33). RK68,69AA and C89A P1b mutants, which are unable to bind siRNAs and to suppress RNA silencing, did not enhance PVX pathogenicity (Fig. R33), indicating that suppression of virus-induced RNA silencing by siRNA binding is the activity of P1b that stimulates PVX infection.

Although the PVX system is a very useful tool, the presence of PVX-specific factors, specially its RSS P25, as well as the absence of additional potyvirid-specific factors, could affect the P1b action. Thus, a homologous potyviral system was developed by replacement of the PPV RSS, HCPro, by P1b (P1P1b, Fig. R34B). Interestingly, whereas a PPV deletion mutant carrying no HCPro (P1ΔHC; Fig. R34B) was unable to infect the herbaceous host *N. benthamiana* (Fig. R36), the generated chimerical virus was infectious and accumulated at high levels (Fig. R35), indicating that P1b is able to replace HCPro during a potyviral infection, and that PPV does not depend on exclusive functions of HCPro for the infection process.

Diverse engineered viruses lacking their RSSs have been designed and well-characterized, as tombusviruses CymRSV and TBSV lacking P19 (Omarov *et al.*, 2006; Omarov *et al.*, 2007; Qiu *et al.*, 2002; Szittya *et al.*, 2002) and 2b-deletion mutants of the cucumovirus CMV (Diaz-Pendon *et al.*, 2007; Soards *et al.*, 2002; Ziebell & Carr, 2009; Ziebell *et al.*, 2007). These studies have shown that these viruses are able to initiate the infection process in different hosts, reaching till upper-non inoculated leaves, but exhibiting attenuated infections characterized by milder symptoms and "recovery" phenotypes, caused by antiviral RNA silencing responses. In contrast, the P1ΔHC virus, as well as chimerical P1P1b viruses carrying anti-silencing defective P1b versions, is completely unable to initiate an infection process (Fig. R36), demonstrating the key relevance of an active RSS for potyviral viability. This strict requirement was also recently reported for an RSS-deficient TuMV expressing an inactive version of HCPro, which only infected *N. benthamiana* when the tombusviral RSS P19 was exogenously supplied (Garcia-Ruiz *et al.*, 2010). Moreover, whereas TuMV-susceptible *A. thaliana* plants turned immune to the HCPro-deficient virus, the immunity was lost in mutant plants lacking main components of the RNA silencing machinery (Garcia-Ruiz *et al.*, 2010), highlighting again the importance of counteracting antiviral silencing for potyviral infections. Several reasons might explain the different level of requirement for RNA silencing suppression factors of potyviruses and other viruses, even in the same experimental hosts: i) higher rates of replication and movement through de plant could allow some viruses to escape antiviral RNA silencing more easily, ii) although all the compared viruses replicate in the cytoplasm their genomic RNAs could differ in accessibility by the silencing effector machinery, iii) the larger size of the genomic potyviral RNAs could make them more susceptible to the RNA silencing action, iv) finally, the possibility that other viral factors could provide some antiviral activity that partially compensate the absence of the main RSS in some viral systems cannot be ruled out.

It is also worth to remark that although potyvirid have an strict requirement for RNA silencing suppression, this activity can be provided by different viral proteins: HCPro in potyviruses and P1b in ipomoviruses and tritimoviruses. Interestingly, whereas HCPro is the RSS of member of the *Potyvirus* genus, in members of other genera of the family *Potyviridae* having HCPro and a P1b-like P1, HCPro lacks RNA silencing suppression activity (Giner *et al.*, 2010; Stenger *et al.*, 2007). This would be compatible with previous suggestions that evolution has provided plant viruses with RNA silencing suppression activity very recently, adapting very different viral proteins to the novel job (Voinnet, 2005). All experimental evidences available indicate that HCPro and CVYV P1b suppress the RNA silencing by

sequestering small RNAs. However, this mechanism appears not to be universal for P1b-like P1s. Whereas *in vitro* assays suggest that the P1 protein of the type member of the *Ipomovirus* genus SPMMV does not bind small RNAs, this protein suppresses silencing by binding to AGO1 and inhibition of RISC activity (Giner *et al.*, 2010). The existence of two different ways to counteract silencing for P1b-like P1 proteins suggests that these proteins could acquired their silencing suppression activities in independent evolutionary events, after splitting of the lineages driving to the different P1b-P1-containing potyvirid (Fig. D1). However, the conservation in all P1b-like P1s of the LxKA motif (Fig. R30), which is involved in silencing suppression mediated by siRNA binding, might suggests that this mechanism of silencing suppression was already adopted by the common ancestor of all P1b-like P1s, and was then lost in some lineages after acquisition of a different silencing suppression strategy. Alternatively, the LxKA domain could be broadly conserved because it is involved in a primitive, still unknown, function of P1b-like P1s, and it was co-opted for the acquisition of RNA silencing suppression in one of the P1b-like P1 lineages.

Another still open question is the relationship of classical P1 proteins and RNA silencing. As I mentioned above, whereas no silencing suppression activity has been observed for P1 potyviral proteins in standard agroinfiltration systems, a number of observations suggest that this proteins could enhance the silencing suppression activity of HCPro (Anandalakshmi *et al.*, 1998; Kasschau & Carrington, 1998; Pruss *et al.*, 1997; Rajamäki *et al.*, 2005). Recent results from our laboratory show that in contrast with the lethal effect of the ΔHCPro mutation, the ΔP1 mutation does not abolish PPV infectivity, as it had been also shown previously for TEV (Verchot & Carrington, 1995). However, the phenotype of the PPV ΔP1 mutant suggests that the ΔP1 deletion migth cause a defect in RNA silencing suppression (unpublished results). This phenotype could be compatible both with the proposed enhancer effect of P1 on HCPro activity and with an independent silencing suppression activity of P1 that only works during viral infection. It is important to remark that other potyviral proteins could be contributing to counteract anti-viral silencing. In this regard, a weak silencing suppression activity has been recently reported for the VPg protein of the potyvirus PVA (Rajamaki & Valkonen, 2009).

It will be very interesting to unravel in the future how, in one hand, very different proteins are able to perform similar RNA silencing suppression functions, and, on the other hand, how closely related silencing suppressors have adopted quite different suppression mechanisms that are used to neutralize antiviral silencing by very similar viruses. Moreover, if the P1b-like P1s play additional roles independently of silencing suppression, as HCPro

does, studies of the structural and functional relationships of these activities will be an exciting research challenge that will help us not only to understand the functions of these proteins, but also the complex evolution of viruses from family *Potyviridae*.

Conclusions

V. CONCLUSIONS

1- Recombination and gene duplication events have contributed to P1 diversification, and they could have helped to successful adaptation of viruses from the family *Potyviridae* to a wide range of host species.

2- P1 proteins from members of the family *Potyviridae* can be classified in two groups, classical and P1b-like, which appears to reflect not only sequence divergence, but also by functional diversification.

3- The CVYV genome encodes two active P1 serine proteases that cleavage their C-terminus, named P1a and P1b, which are evolutively sorted into classical and P1b-like groups, respectively.

4- The CVYV P1b protein has RNA silencing suppression activity, which does not depend on its serine protease activity. In contrast, two conserved domains, a basic motif with an LxKA signature and a putative zinc finger, play key roles in the anti-silencing activity of CVYV P1b.

5- The CVYV P1b proteins interact with itself *in planta*, probably forming homodimers. The zinc finger domain is involved in protein-protein interactions keeping a proper oligomer conformation.

6- CVYV P1b resembles potyviral HCPro in binding double-stranded siRNAs with preference for 21-nt size, but it differs from potyviral HCPro in binding siRNAs without 5' phosphate or 3' 2-nt overhangs.

7- Sequestration of siRNAs is proposed as the molecular mechanism of CVYV P1b to suppress RNA silencing. The LxKA motif forms part of the small RNA binding domain of the protein.

8- The expression of CVYV P1b alters the size profile of endogenous small RNA. Different populations of small RNAs, including siRNAs and miRNAs, are specifically bound by the viral factor.

9- Wild type CVYV P1b, but not anti-silencing-defective mutants, is able to functionally replace PPV HCPro, indicating that, whereas potyviral infection does not dependent on a particular silencing suppressor, it has an strict requirement for RNA silencing suppression activity.

1. Eventos de recombinación y duplicación génica han participado en la diversificación de la proteína P1 en la familia *Potyviridae*, y podrían haber ayudado a la exitosa adaptación de los virus de esta familia a un amplio espectro de huéspedes.

2. Las proteínas P1 de los virus de la familia *Potyviridae* se pueden clasificar en dos grupos, "clásicas" y "tipo P1b". Esta clasificación parece reflejar no sólo divergencia de secuencia sino también diversificación funcional.

3. El genoma de CVYV codifica a dos serín proteasas P1, P1a y P1b, que se auto-cortan en su extremo C-terminal. P1a pertenece al grupo de las proteínas P1 "clásicas" y P1b se puede considerar como el prototipo de las proteínas "tipo P1b".

4. La proteína P1b de CVYV tiene actividad supresora del silenciamiento de RNA, que no precisa de su actividad proteolítica. Por el contrario, un dominio básico caracterizado por una secuencia LxKA y un posible dominio "zinc finger", ambos parcialmente conservados en las proteinas "tipo P1b", desempeñan funciones clave para la actividad anti-silenciamiento de la proteína P1b de CVYV

5. La proteína P1b de CVYV interacciona consigo misma *in planta*, formando probablemente homodímeros. El dominio "zinc finger" está implicado en interacciones proteína-proteína que permiten un correcto acoplamiento del oligómero de P1b.

6. La proteína P1b de CVYV se parece a la proteína HCPro de los potyvirus en que une siRNAs de doble cadena con preferencia por las moléculas de 21 nt. Por el contrario, P1b se diferencia de HCPro en que es capaz de unirse a siRNAs que carecen de fosfato en el extremo 5' y de protuberancias de 2 nt en el extremo 3'

7. Los resultados de esta tesis sugieren fuertemente que el mecanismo molecular que emplea la proteína P1b de CVYV para suprimir el silenciamiento de RNA es el secuestro e inactivación de los siRNAs virales. El dominio LxKA forma parte de la región de unión a siRNAs de la proteína.

8. La expresión de P1b de CVYV altera el perfil de tamaño de los pequeños RNAs endógenos de la planta. P1b se une a diferentes poblaciones de pequeños RNAs, incluidos siRNAs y miRNAs.

9. La proteína P1b de CVYV silvestre, pero no mutantes de ella carentes de actividad anti-silenciamiento, es capaz de reemplazar funcionalmente a la proteína HCPro de PPV, lo que permite concluir que la infección potyviral no depende de un supresor de silenciamiento específico pero tiene un requerimiento estricto de actividad supresora de silenciamiento.

References

VI. REFERENCES

Adams, M. J., Antoniw, J. F. & Beaudoin, F. (2005a). Overview and analysis of the polyprotein cleavage sites in the family *Potyviridae*. *Mol Plant Pathol* **6**, 471-487.

Adams, M. J., Antoniw, J. F. & Fauquet, C. M. (2005b). Molecular criteria for genus and species discrimination within the family *Potyviridae*. *Arch Virol* **150**, 459-479.

Adams, M. J., Antoniw, J. F. & Mullins, J. G. L. (2001). Plant virus transmission by plasmodiophorid fungi is associated with distinctive transmembrane regions of virus-encoded proteins. *Arch Virol* **146**, 1139-1153.

Ali, A., Natsuaki, T. & Okuda, S. (2006). The complete nucleotide sequence of a Pakistani isolate of *Watermelon mosaic virus* provides further insights into the taxonomic status in the *Bean common mosaic virus* subgroup. *Virus Genes* **32**, 307-311.

Anandalakshmi, R., Marathe, R., Ge, X., Herr Jr., J. M., Mau, C., Mallory, A., Pruss, G., Bowman, L. & Vance, V. B. (2000). A calmodulin-related protein that suppresses posttranscriptional gene silencing in plants. *Science* **290**, 142-144.

Anandalakshmi, R., Pruss, G. J., Ge, X., Marathe, R., Mallory, A. C., Smith, T. H. & Vance, V. B. (1998). A viral suppressor of gene silencing in plants. *Proc Natl Acad Sci USA* **95**, 13079-13084.

Anindya, R., Chittori, S. & Savithri, H. S. (2005). Tyrosine 66 of Pepper vein banding virus genome-linked protein is uridylylated by RNA-dependent RNA polymerase. *Virology* **336**, 154-162.

Anindya, R. & Savithri, H. S. (2004). Potyviral NIa proteinase, a proteinase with novel deoxyribonuclease activity. *J Biol Chem* **279**, 32159-32169.

Arazi, T., Shiboleth, Y. M. & Gal-On, A. (2001). A nonviral peptide can replace the entire N terminus of zucchini yellow mosaic potyvirus coat protein and permits viral systemic infection. *J Virol* **75**, 6329-6336.

Atreya, C. D. & Pirone, T. P. (1993). Mutational analysis of the helper component-proteinase gene of a potyvirus: Effects of amino acid substitutions, deletions, and gene replacement on virulence and aphid transmissibility. *Proc Natl Acad Sci USA* **90**, 11919-11923.

Azevedo, J., Garcia, D., Pontier, D., Ohnesorge, S., Yu, A., Garcia, S., Braun, L., Bergdoll, M., Hakimi, M. A., Lagrange, T. & Voinnet, O. (2010). Argonaute quenching and global changes in Dicer homeostasis caused by a pathogen-encoded GW repeat protein. *Genes Dev* **24**, 904-915.

Bateson, M. F., Lines, R. E., Revill, P., Chaleeprom, W., Ha, C. V., Gibbs, A. J. & Dale, J. L. (2002). On the evolution and molecular epidemiology of the potyvirus Papaya ringspot virus. *J Gen Virol* **83**, 2575-2585.

Baulcombe, D. (2005). RNA silencing. *Trends Biochem Sci* **30**, 290-293.

Baulcombe, D. C. & Molnár, A. (2004). Crystal structure of p19 - a universal suppressor of RNA silencing. *Trends Biochem Sci* **29**, 279-281.

Baumberger, N. & Baulcombe, D. C. (2005). *Arabidopsis* ARGONAUTE1 is an RNA Slicer that selectively recruits microRNAs and short interfering RNAs. *Proc Natl Acad Sci USA* **102**, 11928-11933.

Baumberger, N., Tsai, C. H., Lie, M., Havecker, E. & Baulcombe, D. C. (2007). The polerovirus silencing suppressor P0 targets ARGONAUTE proteins for degradation. *Curr Biol* **17**, 1609-1614.

Bayne, E. H., Rakitina, D. V., Morozov, S. Y. & Baulcombe, D. C. (2005). Cell-to-cell movement of *Potato Potexvirus X* is dependent on suppression of RNA silencing. *Plant J* **44**, 471-482.

Béclin, C., Berthomé, R., Palauqui, J.-C., Tepfer, M. & Vaucheret, H. (1998). Infection of tobacco or *Arabidopsis* plants by CMV counteracts systemic post-transcriptional silencing of nonviral (Trans)genes. *Virology* **252**, 313-317.

Berger, P. H., Adams, M. J., Brunt, A. A., Hammond, J., Hill, J. H., Jordan, R. L., Kashiwazalci, S., Rybicki, E., Spence, N. & Stenger, D. C. (2005). Family Potyviridae. In: Fauquet, C. M., Mayo, M.A., Maniloff, J. Desselberger, U., and Ball, L.A. (eds). *Taxonomy of Viruses The International Committee on the Taxonomy of Viruses, 8th Report Elsevier/Academic Press, London*, 819-841.

Bilgin, D. D., Liu, Y., Schiff, M. & Dinesh-Kumar, S. P. (2003). $P58^{IPK}$, a plant ortholog of double-stranded RNA-dependent protein kinase PKR inhibitor, functions in viral pathogenesis. *Dev Cell* **4**, 651-661.

Blanc, S., LopezMoya, J. J., Wang, R. Y., GarciaLampasona, S., Thornbury, D. W. & Pirone, T. P. (1997). A specific interaction between coat protein and helper component correlates with aphid transmission of a potyvirus. *Virology* **231**, 141-147.

Blevins, T., Rajeswaran, R., Shivaprasad, P. V., Beknazariants, D., Si-Ammour, A., Park, H. S., Vazquez, F., Robertson, D., Meins, F., Jr., Hohn, T. & Pooggin, M. M. (2006). Four plant Dicers mediate viral small RNA biogenesis and DNA virus induced silencing. *Nucleic Acids Res* **34**, 6233-6246.

Borsani, O., Zhu, J. H., Verslues, P. E., Sunkar, R. & Zhu, J. K. (2005). Endogenous siRNAs derived from a pair of natural cis-antisense transcripts regulate salt tolerance in *Arabidopsis*. *Cell* **123**, 1279-1291.

Bortolamiol, D., Pazhouhandeh, M., Marrocco, K., Genschik, P. & Ziegler-Graff, V. (2007). The polerovirus F box protein P0 targets ARGONAUTE1 to suppress RNA silencing. *Curr Biol* **17**, 1615-1621.

Bosse, G. D. & Simard, M. J. (2010). A new twist in the microRNA pathway: Not Dicer but Argonaute is required for a microRNA production. *Cell Res* **20**, 735-737.

Bouché, N., Lauressergues, D., Gasciolli, V. & Vaucheret, H. (2006). An antagonistic function for Arabidopsis DCL2 in development and a new function for DCL4 in generating viral siRNAs. *EMBO J* **25**, 3347-3356.

Bousalem, M., Douzery, E. J. & Fargette, D. (2000). High genetic diversity, distant phylogenetic relationships and intraspecies recombination events among natural populations of Yam mosaic virus: a contribution to understanding potyvirus evolution. *J Gen Virol* **81**, 243-255.

Bragg, J. N. & Jackson, A. O. (2004). The C-terminal region of the *Barley stripe mosaic virus* γb protein participates in homologous interactions and is required for suppression of RNA silencing. *Mol Plant Pathol* **5**, 465-481.

Brennecke, J., Aravin, A. A., Stark, A., Dus, M., Kellis, M., Sachidanandam, R. & Hannon, G. J. (2007). Discrete small RNA-generating loci as master regulators of transposon activity in Drosophila. *Cell* **128**, 1089-1103.

Brigneti, G., Voinnet, O., Li, W. X., Ji, L. H., Ding, S. W. & Baulcombe, D. C. (1998). Viral pathogenicity determinants are suppressors of transgene silencing in *Nicotiana benthamiana*. *EMBO J* **17**, 6739-6746.

Brodersen, P., Sakvarelidze-Achard, L., Bruun-Rasmussen, M., Dunoyer, P., Yamamoto, Y. Y., Sieburth, L. & Voinnet, O. (2008). Widespread translational inhibition by plant miRNAs and siRNAs. *Science* **320**, 1185-1190.

Bucher, E., Hemmes, H., de Haan, P., Goldbach, R. & Prins, M. (2004). The influenza A virus NS1 protein binds small interfering RNAs and suppresses RNA silencing in plants. *J Gen Virol* **85**, 983-991.

Buck, K. W. (1996). Comparison of the replication of positive-stranded RNA viruses of plants and animals. In *Advances in Virus Research, V*, pp. 159-251. Edited by K. Maramorosch, F. A. Murphy & A. J. Shatkin. San Diego, CA: Academic Press Inc.

Burd, C. G. & Dreyfuss, G. (1994). Conserved structures and diversity of functions of RNA-binding proteins. *Science* **265**, 615-621.

Burgyán, J. (2008). Role of silencing suppressor proteins. *Methods Mol Biol* **451**, 69-79.

Cañizares, M. C., Navas-Castillo, J. & Moriones, E. (2008). Multiple suppressors of RNA silencing encoded by both genomic RNAs of the crinivirus, *Tomato chlorosis virus*. *Virology*.

Carbonell, A., de Alba, A. E. M., Flores, R. & Gago, S. (2008). Double-stranded RNA interferes in a sequence-specific manner with the infection of representative members of the two viroid families. *Virology* **371**, 44-53.

Carrington, J. C. & Freed, D. D. (1990). Cap-independent enhancement of translation by a plant potyvirus 5' nontranslated region. *J Virol* **64**, 1590-1597.

Carrington, J. C., Freed, D. D. & Leinicke, A. J. (1991). Bipartite signal sequence mediates nuclear translocation of the plant potyviral NIa protein. *Plant Cell* **3**, 953-962.

Carrington, J. C., Jensen, P. E. & Schaad, M. C. (1998). Genetic evidence for an essential role for potyvirus CI protein in cell-to-cell movement. *Plant J* **14**, 393-400.

Carrington, J. C., Kasschau, K. D. & Johansen, L. K. (2001). Activation of suppression of RNA silencing by plant viruses. *Virology* **281**, 1-5.

Cervera, M. T., Riechmann, J. L., Martin, M. T. & García, J. A. (1993). 3'-Terminal sequence of the plum pox virus PS and o6 isolates: Evidence for RNA recombination within the potyvirus group. *J Gen Virol* **74**, 329-334.

Chao, J. A., Lee, J. H., Chapados, B. R., Debler, E. W., Schneemann, A. & Williamson, J. R. (2005). Dual modes of RNA-silencing suppression by flock house virus protein B2. *Nat Struct Mol Biol* **12**, 952-957.

Chapman, E. J. & Carrington, J. C. (2007). Specialization and evolution of endogenous small RNA pathways. *Nature Reviews Genetics* **8**, 884-896.

Chapman, E. J., Prokhnevsky, A. I., Gopinath, K., Dolja, V. V. & Carrington, J. C. (2004). Viral RNA silencing suppressors inhibit the microRNA pathway at an intermediate step. *Genes Dev* **18**, 1179-1186.

Chare, E. R. & Holmes, E. C. (2006). A phylogenetic survey of recombination frequency in plant RNA viruses. *Arch Virol* **151**, 933-946.

Charron, C., Nicolai, M., Gallois, J. L., Robaglia, C., Moury, B., Palloix, A. & Caranta, C. (2008). Natural variation and functional analyses provide evidence for co-evolution between plant eIF4E and potyviral VPg. *Plant J* **54**, 56-68.

Chellappan, P., Vanitharani, R. & Fauquet, C. M. (2004). Short interfering RNA accumulation correlates with host recovery in DNA virus-infected hosts, and gene silencing targets specific viral sequences. *J Virol* **78**, 7465-7477.

Chellappan, P., Vanitharani, R. & Fauquet, C. M. (2005). MicroRNA-binding viral protein interferes with Arabidopsis development. *Proc Natl Acad Sci USA* **102**, 10381-10386.

Chen, D., Juárez, S., Hartweck, L., Alamillo, J. M., Simón-Mateo, C., Pérez, J. J., Fernández-Fernández, M. R., Olszewski, N. E. & García, J. A. (2005). Identification of secret agent as the *O*-GlcNAc transferase that participates in Plum Pox virus infection. *J Virol* **79**, 9381-9387.

Chen, H. M., Chen, L. T., Patel, K., Li, Y. H., Baulcombe, D. C. & Wu, S. H. (2010). 22-nucleotide RNAs trigger secondary siRNA biogenesis in plants. *Proc Natl Acad Sci U S A*.

Chen, H. Y., Yang, J., Lin, C. Q. & Adam Yuan, Y. (2008). Structural basis for RNA-silencing suppression by *Tomato aspermy virus* protein 2b. *EMBO Rep* **9**, 754-760.

Chen, J., Li, W. X., Xie, D. X., Peng, J. R. & Ding, S. W. (2004a). Viral virulence protein suppresses RNA silencing-mediated defense but upregulates the role of MicroRNA in host gene expression. *Plant Cell* **16**, 1302-1313.

Chen, J., Zheng, H. Y., Lin, L., Adams, M. J., Antoniw, J. F., Zhao, M. F., Shang, Y. F. & Chen, J. P. (2004b). A virus related to *Soybean mosaic virus* from *Pinellia ternata* in China and its comparison with local soybean SMV isolates. *Arch Virol* **149**, 349-363.

Chen, X. (2009). Small RNAs and their roles in plant development. *Annual review of cell and developmental biology* **25**, 21-44.

Cheng, Y. Q., Liu, Z. M., Xu, J., Zhou, T., Wang, M., Chen, Y. T., Li, H. F. & Fan, Z. F. (2008). HC-Pro protein of sugar cane mosaic virus interacts specifically with maize ferredoxin-5 in vitro and in planta. *J Gen Virol* **89**, 2046-2054.

Chiang, C. H., Lee, C. Y., Wang, C. H., Jan, F. J., Lin, S. S., Chen, T. C., Raja, J. A. J. & Yeh, S. D. (2007). Genetic analysis of an attenuated *Papaya ringspot virus* strain applied for cross-protection. *Eur J Plant Pathol* **118**, 333-348.

Chiba, M., Reed, J. C., Prokhnevsky, A. I., Chapman, E. J., Mawassi, M., Koonin, E. V., Carrington, J. C. & Dolja, V. V. (2006). Diverse suppressors of RNA silencing enhance agroinfection by a viral replicon. *Virology* **346**, 7-14.

Chiu, M. H., Chen, I. H., Baulcombe, D. C. & Tsai, C. H. (2010). The silencing suppressor P25 of Potato virus X interacts with Argonaute1 and mediates its degradation through the proteasome pathway. *Mol Plant Pathol* **11**, 641-649.

Chung, B. Y. W., Miller, W. A., Atkins, J. F. & Firth, A. E. (2008). An overlapping essential gene in the Potyviridae. *Proc Natl Acad Sci USA* **105**, 5897-5902.

Collins, R. E. & Cheng, X. (2005). Structural domains in RNAi. *Febs Lett* **579**, 5841-5849.

Cotton, S., Grangeon, R., Thivierge, K., Mathieu, I., Ide, C., Wei, T. Y., Wang, A. M. & Laliberte, J. F. (2009). Turnip mosaic virus RNA replication complex vesicles are mobile, align with microfilaments, and are each derived from a single viral genome. *J Virol* **83**, 10460-10471.

Covey, S. N., Al-Kaff, N., Lángara, A. & Turner, D. S. (1997). Plants combat infection by gene silencing. *Nature* **385**, 781-782.

Cox, E. H. & McLendon, G. L. (2000). Zinc-dependent protein folding. *Curr Opin Chem Biol* **4**, 162-165.

Csorba, T., Bovi, A., Dalmay, T. & Burgyán, J. (2007). The p122 subunit of *Tobacco mosaic virus* replicase is a potent silencing suppressor and compromises both small interfering RNA- and MicroRNA-mediated pathways. *J Virol* **81**, 11768-11780.

Csorba, T., Lozsa, R., Hutvagner, G. & Burgyan, J. (2010). Polerovirus protein P0 prevents the assembly of small RNA-containing RISC complexes and leads to degradation of ARGONAUTE1. *Plant J* **62**, 463-472.

Cuellar, W. J., Kreuze, J. F., Rajamaki, M. L., Cruzado, K. R., Untiveros, M. & Valkonen, J. P. (2009). Elimination of antiviral defense by viral RNase III. *Proc Natl Acad Sci USA* **106**, 10354-10358.

Cuperus, J. T., Carbonell, A., Fahlgren, N., Garcia-Ruiz, H., Burke, R. T., Takeda, A., Sullivan, C. M., Gilbert, S. D., Montgomery, T. A. & Carrington, J. C. (2010). Unique functionality of 22-nt miRNAs in triggering RDR6-dependent siRNA biogenesis from target transcripts in Arabidopsis. *Nat Struct Mol Biol.*

Curtin, S. J., Watson, J. M., Smith, N. A., Eamens, A. L., Blanchard, C. L. & Waterhouse, P. M. (2008). The roles of plant dsRNA-binding proteins in RNAi-like pathways. *FEBS Lett* **582**, 2753-2760.

Daròs, J. A., Schaad, M. C. & Carrington, J. C. (1999). Functional analysis of the interaction between VPg-proteinase (NIa) and RNA polymerase (NIb) of tobacco etch potyvirus, using conditional and suppressor mutants. *J Virol* **73**, 8732-8740.

Decroocq, V., Salvador, B., Sicard, O., Glasa, M., Cosson, P., Svanella-Dumas, L., Revers, F., Garcia, J. A. & Candresse, T. (2009). The determinant of potyvirus ability to overcome the RTM resistance of *Arabidopsis thaliana* maps to the N-terminal region of the coat protein. *Mol Plant-Microbe Interact* **22**, 1302-1311.

Deleris, A., Gallego-Bartolome, J., Bao, J. S., Kasschau, K. D., Carrington, J. C. & Voinnet, O. (2006). Hierarchical action and inhibition of plant Dicer-like proteins in antiviral defense. *Science* **313**, 68-71.

Desbiez, C. & Lecoq, H. (2004). The nucleotide sequence of Watermelon mosaic virus (WMV, Potyvirus) reveals interspecific recombination between two related potyviruses in the 5' part of the genome. *Arch Virol* **149**, 1619-1632.

Desprez, T., Juraniec, M., Crowell, E. F., Jouy, H., Pochylova, Z., Parcy, F., Hofte, H., Gonneau, M. & Vernhettes, S. (2007). Organization of cellulose synthase complexes involved in primary cell wall synthesis in Arabidopsis thaliana. *Proc Natl Acad Sci U S A* **104**, 15572-15577.

Diaz-Pendon, J. A. & Ding, S. W. (2008). Direct and indirect roles of viral suppressors of RNA silencing in pathogenesis. *Annu Rev Phytopathol* **46**, 303-326.

Diaz-Pendon, J. A., Li, F., Li, W. X. & Ding, S. W. (2007). Suppression of antiviral silencing by cucumber mosaic virus 2b protein in Arabidopsis Is Associated with drastically reduced accumulation of three classes of viral small interfering RNAs. *Plant Cell* **19**, 2053-2063.

Ding, S. W. & Voinnet, O. (2007). Antiviral immunity directed by small RNAs. *Cell* **130**, 413-426.

Dolgosheina, E. V., Morin, R. D., Aksay, G., Sahinalp, S. C., Magrini, V., Mardis, E. R., Mattsson, J. & Unrau, P. J. (2008). Conifers have a unique small RNA silencing signature. *RNA-Publ RNA Soc* **14**, 1508-1515.

Dolja, V. V., Haldeman-Cahill, R., Montgomery, A. E., Vandenbosch, K. A. & Carrington, J. C. (1995). Capsid protein determinants involved in cell-to-cell and long distance movement of tobacco etch potyvirus. *Virology* **206**, 1007-1016.

Donaire, L., Barajas, D., Martinez-Garcia, B., Martinez-Priego, L., Pagan, I. & Llave, C. (2008). Structural and genetic requirements for the biogenesis of *Tobacco rattle virus*-derived small interfering RNAs. *J Virol* **82**, 5167-5177.

Dong, Z., Han, M. H. & Fedoroff, N. (2008). The RNA-binding proteins HYL1 and SE promote accurate in vitro processing of pri-miRNA by DCL1. *Proc Natl Acad Sci USA* **105**, 9970-9975.

Dorokhov, Y. L. (2007). Gene silencing in plants. *Molecular Biology* **41**, 519-530.

Dougherty, W. G. & Parks, T. D. (1995). Transgenes and gene suppression: telling us something new? *Curr Opin Cell Biol* **7**, 399-405.

Du, Q. S., Duan, C. G., Zhang, Z. H., Fang, Y. Y., Fang, R. X., Xie, Q. & Guo, H. S. (2007). DCL4 targets *Cucumber mosaic virus* satellite RNA at novel secondary structures. *J Virol* **81**, 9142-9151.

Duan, C. G., Wang, C. H., Fang, R. X. & Guo, H. S. (2008). Artificial MicroRNAs highly accessible to targets confer efficient virus resistance in plants. *J Virol* **82**, 11084-11095.

Dunoyer, P., Himber, C., Ruiz-Ferrer, V., Alioua, A. & Voinnet, O. (2007). Intra- and intercellular RNA interference in Arabidopsis thaliana requires components of the microRNA and heterochromatic silencing pathways. *Nature Genetics* **39**, 848-856.

Dunoyer, P., Himber, C. & Voinnet, O. (2005). DICER-LIKE 4 is required for RNA interference and produces the 21-nucleotide small interfering RNA component of the plant cell-to-cell silencing signal. *Nature Genetics* **37**, 1356-1360.

Dunoyer, P., Lecellier, C. H., Parizotto, E. A., Himber, C. & Voinnet, O. (2004). Probing the microRNA and small Interfering RNA pathways with virus-encoded suppressors of RNA silencing. *Plant Cell* **16**, 1235-1250.

Dunoyer, P., Schott, G., Himber, C., Meyer, D., Takeda, A., Carrington, J. C. & Voinnet, O. (2010). Small RNA duplexes function as mobile silencing signals between plant cells. *Science* **328**, 912-916.

Dunoyer, P. & Voinnet, O. (2008). Mixing and matching: the essence of plant systemic silencing? *Trends Genet* **24**, 151-154.

Ebhardt, H. A., Thi, E. P., Wang, M. B. & Unrau, P. J. (2005). Extensive 3' modification of plant small RNAs is modulated by helper component-proteinase expression. *Proc Natl Acad Sci USA* **102**, 13398-13403.

El-Shami, M., Pontier, D., Lahmy, S., Braun, L., Picart, C., Vega, D., Hakimi, M. A., Jacobsen, S. E., Cooke, R. & Lagrange, T. (2007). Reiterated WG/GW motifs form functionally and evolutionarily conserved ARGONAUTE-binding platforms in RNAi-related components. *Genes Dev* **21**, 2539-2544.

Endres, M. W., Gregory, B. D., Gao, Z., Foreman, A. W., Mlotshwa, S., Ge, X., Pruss, G. J., Ecker, J. R., Bowman, L. H. & Vance, V. (2010). Two plant viral suppressors of silencing require the ethylene-inducible host transcription factor RAV2 to block RNA silencing. *PLoS pathogens* **6**, e1000729.

English, J. J., Mueller, E. & Baulcombe, D. C. (1996). Suppression of virus accumulation in transgenic plants exhibiting silencing of nuclear genes. *Plant Cell* **8**, 179-188.

Farazi, T. A., Juranek, S. A. & Tuschl, T. (2008). The growing catalog of small RNAs and their association with distinct Argonaute/Piwi family members. *Development* **135**, 1201-1214.

Fenner, B. J., Goh, W. & Kwang, J. (2007). Dissection of double-stranded RNA binding protein B2 from betanodavirus. *J Virol* **81**, 5449-5459.

Fernández, A., Guo, H. S., Sáenz, P., Simón-Buela, L., Gómez de Cedrón, M. & García, J. A. (1997). The motif V of plum pox potyvirus CI RNA helicase is involved in NTP hydrolysis and is essential for virus RNA replication. *Nucleic Acids Res* **25**, 4474-4480.

Fernández, A., Laín, S. & García, J. A. (1995). RNA helicase activity of the plum pox potyvirus CI protein expressed in *Escherichia coli*. Mapping of an RNA binding domain. *Nucleic Acids Res* **23**, 1327-1332.

Fernández-Fernández, M. R., Mouriño, M., Rivera, J., Rodríguez, F., Plana-Durán, J. & García, J. A. (2001). Protection of rabbits against rabbit hemorrhagic disease virus by immunization with the VP60 protein expressed in plants with a potyvirus-based vector. *Virology* **280**, 283-291.

Förstemann, K., Horwich, M. D., Wee, L., Tomari, Y. & Zamore, P. D. (2007). *Drosophila* microRNAs are sorted into functionally distinct argonaute complexes after production by Dicer-1. *Cell* **130**, 287-297.

Frank, F., Sonenberg, N. & Nagar, B. (2010). Structural basis for 5'-nucleotide base-specific recognition of guide RNA by human AGO2. *Nature* **465**, 818-822.

Fusaro, A. F., Matthew, L., Smith, N. A., Curtin, S. J., Dedic-Hagan, J., Ellacott, G. A., Watson, J. M., Wang, M. B., Brosnan, C., Carroll, B. J. & Waterhouse, P. M. (2006). RNA interference-inducing hairpin RNAs in plants act through the viral defence pathway. *EMBO Rep* **7**, 1168-1175.

Gamsjaeger, R., Liew, C. K., Loughlin, F. E., Crossley, M. & Mackay, J. P. (2007). Sticky fingers: zinc-fingers as protein-recognition motifs. *Trends Biochem Sci* **32**, 63-70.

García, J. A., Cervera, M. T., Riechmann, J. L. & López-Otín, C. (1993). Inhibitory effects of human cystatin C on plum pox potyvirus proteases. *Plant Mol Biol* **22**, 697-701.

García, J. A., Martín, M. T., Cervera, M. T. & Riechmann, J. L. (1992). Proteolytic processing of the plum pox potyvirus polyprotein by the NIa protease at a novel cleavage site. *Virology* **188**, 697-703.

García, J. A. & Simón-Mateo, C. (2006). A micropunch against plant viruses. *Nat Biotech* **24**, 1358-1359.

García-Arenal, F., Fraile, A. & Malpica, J. M. (2003). Variation and evolution of plant virus populations. *International Microbiology* **6**, 225-232.

Garcia-Ruiz, H., Takeda, A., Chapman, E. J., Sullivan, C. M., Fahlgren, N., Brempelis, K. J. & Carrington, J. C. (2010). *Arabidopsis* RNA-dependent RNA polymerases and dicer-like proteins in antiviral defense and small interfering RNA biogenesis during *Turnip mosaic virus* infection. *Plant Cell* **22**, 481-496.

Gasciolli, V., Mallory, A. C., Bartel, D. P. & Vaucheret, H. (2005). Partially redundant functions of Arabidopsis DICER-like enzymes and a role for DCL4 in producing trans-acting siRNAs. *Curr Biol* **15**, 1494-1500.

Gazzani, S., Lawrenson, T., Woodward, C., Headon, D. & Sablowski, R. (2004). A link between mRNA turnover and RNA interference in *Arabidopsis*. *Science* **306**, 1046-1048.

Ghildiyal, M. & Zamore, P. D. (2009). Small silencing RNAs: an expanding universe. *Nature reviews* **10**, 94-108.

Gibbs, A. J. & Ohshima, K. (2010). Potyviruses and the Digital Revolution. *Annu Rev Phytopathol*.

Gibbs, A. J., Ohshima, K., Phillips, M. J. & Gibbs, M. J. (2008). The prehistory of potyviruses: their initial radiation was during the dawn of agriculture. *PLoS ONE* **3**, e2523.

Giner, A., Lakatos, L., Garcia-Chapa, M., Lopez-Moya, J. J. & Burgyan, J. (2010). Viral Protein Inhibits RISC Activity by Argonaute Binding through Conserved WG/GW Motifs. *PLoS Pathog* **6**, e1000996.

Glais, L., Tribodet, M. & Kerlan, C. (2002). Genomic variability in *Potato potyvirus Y* (PVY): evidence that $PVY^{N}W$ and PVY^{NTN} variants are single to multiple recombinants between PVY^{O} and PVY^{N} isolates. *Arch Virol* **147**, 363-378.

Glasa, M., Palkovics, L., Komínek, P., Labonne, G., Pittnerova, S., Kudela, O., Candresse, T. & Šubr, Z. (2004). Geographically and temporally distant natural recombinant isolates of Plum pox virus (PPV) are genetically very similar and form a unique PPV subgroup. *J Gen Virol* **85**, 2671-2681.

Glazov, E., Phillips, K., Budziszewski, G. J., Meins, F. & Levin, J. Z. (2003). A gene encoding an RNase D exonuclease-like protein is required for post-transcriptional silencing in *Arabidopsis*. *Plant J* **35**, 342-349.

Glick, E., Zrachya, A., Levy, Y., Mett, A., Gidoni, D., Belausov, E., Citovsky, V. & Gafni, Y. (2008). Interaction with host SGS3 is required for suppression of RNA silencing by tomato yellow leaf curl virus V2 protein. *Proc Natl Acad Sci USA* **105**, 157-161.

Gómez de Cedrón, M., Osaba, L., López, L. & García, J. A. (2006). Genetic analysis of the function of the plum pox virus CI RNA helicase in virus movement. *Virus Res* **116**, 136-145.

Gonzalez, I., Martinez, L., Rakitina, D. V., Lewsey, M. G., Atencio, F. A., Llave, C., Kalinina, N. O., Carr, J. P., Palukaitis, P. & Canto, T. (2010). Cucumber mosaic virus 2b protein subcellular targets and interactions: their significance to RNA silencing suppressor activity. *Mol Plant Microbe Interact* **23**, 294-303.

Gorbalenya, A. E. & Koonin, E. V. (1989). Viral proteins containing the purine NTP-binding sequence pattern. *Nucleic Acids Res* **17**, 8413-8439.

Goto, K., Kobori, T., Kosaka, Y., Natsuaki, T. & Masuta, C. (2007). Characterization of silencing suppressor 2b of Cucumber mosaic virus based on examination of its small RNA-binding abilities. *Plant Cell Physiol* **48**, 1050-1060.

Gottwein, E. & Cullen, B. R. (2008). Viral and cellular microRNAs as determinants of viral pathogenesis and immunity. *Cell host microbe* **3**, 375-387.

Gunawardane, L. S., Saito, K., Nishida, K. M., Miyoshi, K., Kawamura, Y., Nagami, T., Siomi, H. & Siomi, M. C. (2007). A slicer-mediated mechanism for repeat-associated siRNA 5 ' end formation in *Drosophila*. *Science* **315**, 1587-1590.

Guo, D., Spetz, C., Saarma, M. & Valkonen, J. P. (2003). Two potato proteins, including a novel RING finger protein (HIP1), interact with the potyviral multifunctional protein HCpro. *Mol Plant-Microbe Interact* **16**, 405-410.

Guo, D. Y., Rajamaki, M. L., Saarma, M. & Valkonen, J. P. T. (2001). Towards a protein interaction map of potyviruses: protein interaction matrixes of two potyviruses based on the yeast two-hybrid system. *Journal of General Virology* **82**, 935-939.

Guo, H. S. & Ding, S. W. (2002). A viral protein inhibits the long range signaling activity of the gene silencing signal. *EMBO J* **21**, 398-407.

Gy, I., Gasciolli, V., Lauressergues, D., Morel, J.-B., Gombert, J., Proux, F., Proux, C., Vaucheret, H. & Mallory, A. C. (2007). *Arabidopsis* FIERY1, XRN2, and XRN3 are endogenous RNA silencing suppressors. *Plant Cell* **19**, 3451-3461.

Haas, G., Azevedo, J., Moissiard, G., Geldreich, A., Himber, C., Bureau, M., Fukuhara, T., Keller, M. & Voinnet, O. (2008). Nuclear import of CaMV P6 is required for infection and suppression of the RNA silencing factor DRB4. *EMBO J* **27**, 2102-2112.

Haasnoot, J., de Vries, W., Geutjes, E. J., Prins, M., de Haan, P. & Berkhout, B. (2007). The Ebola virus VP35 protein is a suppressor of RNA silencing. *PLoS Pathogens* **3**, e86.

Hafren, A., Hofius, D., Ronnholm, G., Sonnewald, U. & Makinen, K. (2010). HSP70 and its cochaperone CPIP promote potyvirus infection in *Nicotiana benthamiana* by regulating viral coat protein functions. *Plant Cell* **22**, 523-535.

Haldeman-Cahill, R., Daròs, J. A. & Carrington, J. C. (1998). Secondary structures in the capsid protein coding sequence and 3' nontranslated region involved in amplification of the tobacco etch virus genome. *J Virol* **72**, 4072-4079.

Hall, K. B. (2002). RNA-protein interactions. *Curr Opin Struct Biol* **12**, 283-288.

Hamilton, A. J. & Baulcombe, D. C. (1999). A species of small antisense RNA in posttranscriptional gene silencing in plants. *Science* **286**, 950-952.

Hamilton, C. M., Frary, A., Lewis, C. & Tanksley, S. D. (1996). Stable transfer of intact high molecular weight DNA into plant chromosomes. *Proc Natl Acad Sci USA* **93**, 9975-9979.

Hammarström, M., Hellgren, N., van den Berg, S., Berglund, H. & Härd, T. (2002). Rapid screening for improved solubility of small human proteins produced as fusion proteins in *Escherichia coli*. *Protein Science* **11**, 313-321.

Han, J., Lee, Y., Yeom, K. H., Kim, Y. K., Jin, H. & Kim, V. N. (2004). The Drosha-DGCR8 complex in primary microRNA processing. *Genes Dev* **18**, 3016-3027.

Haseloff, J., Siemering, K. R., Prasher, D. C. & Hodge, S. (1997). Removal of a cryptic intron and subcellular localization of green fluorescent protein are required to mark transgenic *Arabidopsis* plants brightly. *Proc Natl Acad Sci USA* **94**, 2122-2127.

Hemmes, H., Lakatos, L., Goldbach, R., Burgyan, J. & Prins, M. (2007). The NS3 protein of *Rice hoja blanca tenuivirus* suppresses RNA silencing in plant and insect hosts by efficiently binding both siRNAs and miRNAs. *RNA* **13**, 1079-1089.

Herlitze, S. & Koenen, M. (1990). A general and rapid mutagenesis method using polymerase chain reaction. *Gene* **91**, 143-147.

Herr, A. J., Molnar, A., Jones, A. & Baulcombe, D. C. (2006). Defective RNA processing enhances RNA silencing and influences flowering of Arabidopsis. *Proc Natl Acad Sci USA* **103**, 10994-15001.

Himber, C., Dunoyer, P., Moissiard, G., Ritzenthaler, C. & Voinnet, O. (2003). Transitivity-dependent and -independent cell-to-cell movement of RNA silencing. *EMBO J* **22**, 4523-4533.

Hiraguri, A., Itoh, R., Kondo, N., Nomura, Y., Aizawa, D., Murai, Y., Koiwa, H., Seki, M., Shinozaki, K. & Fukuhara, T. (2005). Specific interactions between Dicer-like proteins and HYL1/DRB-family dsRNA-binding proteins in Arabidopsis thaliana. *Plant Mol Biol* **57**, 173-188.

Ho, T., Pallett, D., Rusholme, R., Dalmay, T. & Wang, H. (2006). A simplified method for cloning of short interfering RNAs from Brassica juncea infected with Turnip mosaic potyvirus and Turnip crinkle carmovirus. *J Virol Methods* **136**, 217-223.

Ho, T., Wang, H., Pallett, D. & Dalmay, T. (2007). Evidence for targeting common siRNA hotspots and GC preference by plant Dicer-like proteins. *FEBS Lett* **581**, 3267-3272.

Höck, J. & Meister, G. (2008). The Argonaute protein family. *Genome Biology* **9**, 210-218.

Hong, Y. & Hunt, A. G. (1996). RNA polymerase activity catalyzed by a potyvirus-encoded RNA-dependent RNA polymerase. *Virology* **226**, 146-151.

Hong, Y., Levay, K., Murphy, J. F., Klein, P. G., Shaw, J. G. & Hunt, A. G. (1995). A potyvirus polymerase interacts with the viral coat protein and VPg in yeast cells. *Virology* **214**, 159-166.

Horton, R. M., Hunt, H. D., Ho, S. N., Pullen, J. K. & Pease, L. R. (1989). Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. *Gene* **77**, 61-68.

Hu, C. D. & Kerppola, T. K. (2003). Simultaneous visualization of multiple protein interactions in living cells using multicolor fluorescence complementation analysis. *Nat Biotech* **21**, 539-545.

Huang, J., Wang, F., Argyris, E., Chen, K., Liang, Z., Tian, H., Huang, W., Squires, K., Verlinghieri, G. & Zhang, H. (2007). Cellular microRNAs contribute to HIV-1 latency in resting primary CD4+ T lymphocytes. *Nature Med* **13**, 1241-1247.

Hutvágner, G. & Zamore, P. D. (2002). A microRNA in a multiple-turnover RNAi enzyme complex. *Science* **297**, 2056-2060.

Itaya, A., Zhong, X. H., Bundschuh, R., Qi, Y. J., Wang, Y., Takeda, R., Harris, A. R., Molina, C., Nelson, R. S. & Ding, B. (2007). A structured viroid RNA serves as a substrate for dicer-like cleavage to produce biologically active small RNAs but is resistant to RNA-induced silencing complex-mediated degradation. *J Virol* **81**, 2980-2994.

Ivanov, K. I., Puustinen, P., Gabrenaite, R., Vihinen, H., Rönnstrand, L., Valmu, L., Kalkkinen, N. & Mäkinen, K. (2003). Phosphorylation of the potyvirus capsid protein by protein kinase CK2 and its relevance for virus infection. *Plant Cell* **15**, 2124-2139.

Janssen, D., Martín, G., Velasco, L., Gómez, P., Segundo, E., Ruiz, L. & Cuadrado, I. M. (2005). Absence of a coding region for the helper component-proteinase in the genome of cucumber vein yellowing virus, a whitefly-transmitted member of the *Potyviridae*. *Arch Virol* **150**, 1439-1447.

Jiménez, I. (2004). Análisis de las interacciones de la proteína CI del virus de la sharka con proteínas de la planta. In *Escuela Técnica Superior de Ingenieros Agrónomos*: Universidad Politécnica de Madrid.

Jiménez, I., López, L., Alamillo, J. M., Valli, A. & García, J. A. (2006). Identification of a Plum pox virus CI-interacting protein from chloroplast that has a negative effect in virus infection. *Mol Plant-Microbe Interact* **19**, 350-358.

Jin, Y. S., Ma, D. Y., Dong, J. L., Jin, J. C., Li, D. F., Deng, C. W. & Wang, T. (2007a). HC-Pro protein of *Potato Virus Y* can interact with three *Arabidopsis* 20S proteasome subunits in planta. *J Virol* **81**, 12881-12888.

Jin, Y. S., Ma, D. Y., Dong, J. L., Li, D. F., Deng, C. W., Jin, J. C. & Wang, T. (2007b). The HC-Pro protein of Potato virus Y interacts with NtMinD of tobacco. *Mol Plant-Microbe Interact* **20**, 1505-1511.

Jopling, C. L., Yi, M. K., Lancaster, A. M., Lemon, S. M. & Sarnow, P. (2005). Modulation of hepatitis C virus RNA abundance by a liver-specific microRNA. *Science* **309**, 1577-1581.

Kalantidis, K., Schurnacher, H. T., Alexiadis, T. & Helm, J. M. (2008). RNA silencing movement in plants. *Biology of the Cell* **100**, 13-26.

Karlowski, W. M., Zielezinski, A., Carrere, J., Pontier, D., Lagrange, T. & Cooke, R. (2010). Genome-wide computational identification of WG/GW Argonaute-binding proteins in Arabidopsis. *Nucleic Acids Res*.

Kasschau, K. D. & Carrington, J. C. (1995). Requirement for HC-Pro processing during genome amplification of tobacco etch potyvirus. *Virology* **209**, 268-273.

Kasschau, K. D. & Carrington, J. C. (1998). A counterdefensive strategy of plant viruses: Suppression of posttranscriptional gene silencing. *Cell* **95**, 461-470.

Kasschau, K. D. & Carrington, J. C. (2001). Long-distance movement and replication maintenance functions correlate with silencing suppression activity of potyviral HC-Pro. *Virology* **285**, 71-81.

Kasschau, K. D., Xie, Z. X., Allen, E., Llave, C., Chapman, E. J., Krizan, K. A. & Carrington, J. C. (2003). P1/HC-Pro, a viral suppressor of RNA silencing, interferes with *Arabidopsis* development and miRNA function. *Dev Cell* **4**, 205-217.

Katiyar-Agarwal, S., Gao, S., Vivian-Smith, A. & Jin, H. (2007). A novel class of bacteria-induced small RNAs in Arabidopsis. *Genes Dev* **21**, 3123-3134.

Kennedy, S., Wang, D. & Ruvkun, G. (2004). A conserved siRNA-degrading RNase negatively regulates RNA interference in C-elegans. *Nature* **427**, 645-649.

Kiriakidou, M., Tan, G. S., Lamprinaki, S., De Planell-Saguer, M., Nelson, P. T. & Mourelatos, Z. (2007). An mRNA m(7)G cap binding-like motif within human Ago2 represses translation. *Cell* **129**, 1141-1151.

Klug, A. & Rhodes, D. (1987). `Zinc fingers': a novel protein motif for nucleic acid recognition. *Trends Biochem Sci* **12**, 464-469.

Kosakovsky Pond, S. L., Posada, D., Gravenor, M. B., Woelk, C. H. & Frost, S. D. (2006). Automated phylogenetic detection of recombination using a genetic algorithm. *Mol Biol Evol* **23**, 1891-1901.

Krause-Sakate, R., Fakhfakh, H., Peypelut, M., Pavan, M. A., Zerbini, F. M., Marrakchi, M., Candresse, T. & Le Gall, O. (2004). A naturally occurring recombinant isolate of Lettuce mosaic virus. *Arch Virol* **149**, 191-197.

Kreuze, J. F., Savenkov, E. I., Cuellar, W., Li, X. D. & Valkonen, J. P. T. (2005). Viral class 1 RNase III involved in suppression of RNA silencing. *J Virol* **79**, 7227-7238.

Kurihara, Y., Inaba, N., Kutsuna, N., Takeda, A., Tagami, Y. & Watanabe, Y. (2007). Binding of tobamovirus replication protein with small RNA duplexes. *J Gen Virol* **88**, 2347-2352.

Kurihara, Y. & Watanabe, Y. (2004). Arabidopsis micro-RNA biogenesis through Dicer-like 1 protein functions. *Proc Natl Acad Sci USA* **101**, 12753-12758.

Lagrimini, L. M., Bradford, S. & Rothstein, S. (1990). Peroxidase-induced wilting in transgenic tobacco plants. *Plant Cell* **2**, 7-18.

Laín, S., Riechmann, J. L., Martín, M. T. & García, J. A. (1989). Homologous potyvirus and flavivirus proteins belonging to a superfamily of helicase-like proteins. *Gene* **82**, 357-362.

Lakatos, L., Csorba, T., Pantaleo, V., Chapman, E. J., Carrington, J. C., Liu, Y. P., Dolja, V. V., Calvino, L. F., López-Moya, J. J. & Burgyán, J. (2006). Small RNA binding is a common strategy to suppress RNA silencing by several viral suppressors. *EMBO J* **25**, 2768-2780.

Langenberg, W. G. & Zhang, L. Y. (1997). Immunocytology shows the presence of tobacco etch virus P3 protein in nuclear inclusions. *J Struct Biol* **118**, 243-247.

Larsen, R. C., Miklas, P. N., Druffel, K. L. & Wyatt, S. D. (2005). NL-3 K strain Is a stable and naturally occurring interspecific recombinant derived from *Bean common mosaic necrosis virus* and *Bean common mosaic virus*. *Phytopathology* **95**, 1937-1942.

Lecellier, C.-H., Dunoyer, P., Arar, K., Lehmann-Che, J., Eyquem, S., Himber, C., Saïb, A. & Voinnet, O. (2005). A cellular microRNA mediates antiviral defense in human cells. *Science* **308**, 557-560.

Lesemann, D. E. & Winter, S. (2002). Konjac mosaic virus, dasheen mosaic virus and unknown potyviruses infecting *Zantedeschia* Spp. and other cultivated *Araceae*. *Acta Horticul* **568**, 135-141.

Leuschner, P. J. F., Ameres, S. L., Kueng, S. & Martinez, J. (2006). Cleavage of the siRNA passenger strand during RISC assembly in human cells. *EMBO Rep* **7**, 314-320.

Li, F. & Ding, S. W. (2006). Virus counterdefense: Diverse strategies for evading the RNA-silencing immunity. *Annu Rev Microbiol* **60**, 503-531.

Li, W. M., Hilf, M. E., Webb, S. E., Baker, C. A. & Adkins, S. (2008). Presence of P1b and absence of HC-Pro in Squash vein yellowing virus suggests a general feature of the genus *Ipomovirus* in the family *Potyviridae*. *Virus Res* **135**, 213-219.

Li, X. H., Valdez, P., Olvera, R. E. & Carrington, J. C. (1997). Functions of the tobacco etch virus RNA polymerase (NIb): Subcellular transport and protein-protein interaction with VPg/proteinase (NIa). *J Virol* **71**, 1598-1607.

Lian, S. L., Li, S. Q., Abadal, G. X., Pauley, B. A., Fritzler, M. J. & Chan, E. K. L. (2009). The C-terminal half of human Ago2 binds to multiple GW-rich regions of GW182 and requires GW182 to mediate silencing. *RNA* **15**, 804-813.

Lindbo, J. A., Silva-Rosales, L., Proebsting, W. M. & Dougherty, W. G. (1993). Induction of a highly specific antiviral state in transgenic plants: implications for regulation of gene expression and virus resistance. *Plant Cell* **5**, 1749-1759.

Lingel, A., Simon, B., Izaurralde, E. & Sattler, M. (2005). The structure of the flock house virus B2 protein, a viral suppressor of RNA interference, shows a novel mode of double-stranded RNA recognition. *EMBO Rep* **6**, 1149-1155.

Liu, J., Carmell, M. A., Rivas, F. V., Marsden, C. G., Thomson, J. M., Song, J. J., Hammond, S. M., Joshua-Tor, L. & Hannon, G. J. (2004). Argonaute2 Is the catalytic engine of mammalian RNAi. *Science* **305**, 1437-1441.

Liu, J. Z., Blancaflor, E. B. & Nelson, R. S. (2005). The tobacco mosaic virus 126-Kilodalton protein, a constituent of the virus replication complex, alone or within the complex aligns with and traffics along microfilaments. *Plant Physiol* **138**, 1853-1865.

Liu, Q., Rand, T. A., Kalidas, S., Du, F., Kim, H. E., Smith, D. P. & Wang, X. (2003). R2D2, a bridge between the initiation and effector steps of the *Drosophila* RNAi pathway. *Science* **301**, 1921-1925.

Liu, X., Park, J. K., Jiang, F., Liu, Y., McKearin, D. & Liu, Q. H. (2007). Dicer-1, but not Loquacious, is critical for assembly of miRNA-induced silencing complexes. *RNA-Publ RNA Soc* **13**, 2324-2329.

Lobbes, D., Rallapalli, G., Schmidt, D. D., Martin, C. & Clarke, J. (2006). SERRATE: a new player on the plant microRNA scene. *EMBO Rep* **7**, 1052-1058.

López-Moya, J. J. & García, J. A. (2000). Construction of a stable and highly infectious intron-containing cDNA clone of plum pox potyvirus and its use to infect plants by particle bombardment. *Virus Res* **68**, 99-107.

López-Moya, J. J. & Pirone, T. P. (1998). Charge changes near the N terminus of the coat protein of two potyviruses affect virus movement. *J Gen Virol* **79**, 161-165.

López-Moya, J. J., Valli, A. & García, J. A. (2009). Potyviridae. In *Encyclopedia of Life Sciences (ELS)*, p. http://www.els.net/ [DOI: 10.1002/9780470015902.a9780470000755.pub9780470015902]. Chichester: John Wiley & Sons, Ltd.

López-Moya, J. J., Wang, R. Y. & Pirone, T. P. (1999). Context of the coat protein DAG motif affects potyvirus transmissibility by aphids. *J Gen Virol* **80**, 3281-3288.

Lovisolo, O., Hull, R. & Rosler, O. (2003). Coevolution of viruses with hosts and vectors and possible paleontology. *Adv Virus Res* **62**, 325-379.

Lózsa, R., Csorba, T., Lakatos, L. & Burgyán, J. (2008). Inhibition of 3 ' modification of small RNAs in virus-infected plants require spatial and temporal co-expression of small RNAs and viral silencing-suppressor proteins. *Nucleic Acids Res* **36**, 4099-4107.

Lu, R., Folimonov, A., Shintaku, M., Li, W. X., Falk, B. W., Dawson, W. O. & Ding, S. W. (2004). Three distinct suppressors of RNA silencing encoded by a 20-kb viral RNA genome. *Proc Natl Acad Sci USA* **101**, 15742-15747.

Lu, R., Maduro, M., Li, F., Li, H. W., Broitman-Maduro, G., Li, W. X. & Ding, S. W. (2005). Animal virus replication and RNAi-mediated antiviral silencing in *Caenorhabditis elegans*. *Nature* **436**, 1040-1043.

Ma, J. B., Ye, K. & Patel, D. J. (2004). Structural basis for overhang-specific small interfering RNA recognition by the PAZ domain. *Nature* **429**, 318-322.

MacDiarmid, R. M. (2005). RNA silencing in productive virus infections. *Annu Rev Phytopathol* **43**, 523-544.

Mackay, J. P. & Crossley, M. (1998). Zinc fingers are sticking together. *Trends Biochem Sci* **23**, 1-4.

Mallory, A. C., Reinhart, B. J., Bartel, D., Vance, V. B. & Bowman, L. H. (2002). A viral suppressor of RNA silencing differentially regulates the accumulation of short interfering RNAs and micro-RNAs in tobacco. *Proc Natl Acad Sci USA* **99**, 15228-15233.

Martín-Hernández, A. M. & Baulcombe, D. C. (2008). Tobacco rattle virus 16-kilodalton protein encodes a suppressor of RNA silencing that allows transient viral entry in meristems. *J Virol* **82**, 4064-4071.

Martínez-Turiño, S. & Hernández, C. (2009). Inhibition of RNA silencing by the coat protein of *Pelargonium* flower break virus: distinctions from closely related suppressors. *J Gen Virol* **90**, 519-525.

Matranga, C., Tomari, Y., Shin, C., Bartel, D. P. & Zamore, P. D. (2005). Passenger-strand cleavage facilitates assembly of siRNA into Ago2-containing RNAi enzyme Complexes. *Cell* **123**, 607-620.

Mbanzibwa, D. R., Tian, Y. P., Mukasa, S. B. & Valkonen, J. P. T. (2009). *Cassava brown streak virus* (*Potyviridae*) encodes a putative Maf/HAM1 pyrophosphatase implicated in reduction of mutations and a P1 proteinase that suppresses RNA silencing but contains no HC-Pro. *J Virol* **83**, 6934-6940.

Meister, G., Landthaler, M., Patkaniowska, A., Dorsett, Y., Teng, G. & Tuschl, T. (2004). Human Argonaute2 mediates RNA cleavage targeted by miRNAs and siRNAs. *Mol Cell* **15**, 185-197.

Mérai, Z., Kerényi, Z., Kertész, S., Magna, M., Lakatos, L. & Silhavy, D. (2006). Double-stranded RNA binding may be a general plant RNA viral strategy to suppress RNA silencing. *J Virol* **80**, 5747-5756.

Merits, A., Guo, D. Y., Jarvekulg, L. & Saarma, M. (1999). Biochemical and genetic evidence for interactions between potato A potyvirus-encoded proteins P1 and P3 and proteins of the putative replication complex. *Virology* **263**, 15-22.

Mi, S., Cai, T., Hu, Y., Chen, Y., Hodges, E., Ni, F., Wu, L., Li, S., Zhou, H., Long, C., Chen, S., Hannon, G. J. & Qi, Y. (2008). Sorting of small RNAs into Arabidopsis argonaute complexes Is directed by the 5' terminal nucleotide. *Cell* **133**, 116-127.

Miyoshi, K., Tsukumo, H., Nagami, T., Siomi, H. & Siomi, M. C. (2005). Slicer function of *Drosophila* Argonautes and its involvement in RISC formation. *Genes Dev* **19**, 2837-2848.

Mlotshwa, S., Pruss, G. J., Peragine, A., Endres, M. W., Li, J., Chen, X., Poethig, R. S., Bowman, L. H. & Vance, V. (2008a). DICER-LIKE2 plays a primary role in transitive silencing of transgenes in Arabidopsis. *PLoS ONE* **3**, e1755.

Mlotshwa, S., Pruss, G. J. & Vance, V. (2008b). Small RNAs in viral infection and host defense. *Trends Plant Sci* **13**, 375-382.

Moissiard, G., Parizotto, E. A., Himber, C. & Voinnet, O. (2007). Transitivity in Arabidopsis can be primed, requires the redundant action of the antiviral Dicer-like 4 and Dicer-like 2, and is compromised by viral-encoded suppressor proteins. *RNA* **13**, 1268-1278.

Moissiard, G. & Voinnet, O. (2006). RNA silencing of host transcripts by cauliflower mosaic virus requires coordinated action of the four *Arabidopsis* Dicer-like proteins. *Proc Natl Acad Sci USA* **103**, 19593-19598.

Molnár, A., Csorba, T., Lakatos, L., Várallyay, E., Lacomme, C. & Burgyán, J. (2005). Plant virus-derived small interfering RNAs originate predominantly from highly structured single-stranded viral RNAs. *J Virol* **79**, 7812-7818.

Molnar, A., Melnyk, C. W., Bassett, A., Hardcastle, T. J., Dunn, R. & Baulcombe, D. C. (2010). Small silencing RNAs in plants are mobile and direct epigenetic modification in recipient cells. *Science* **328**, 872-875.

Montgomery, T. A., Howell, M. D., Cuperus, J. T., Li, D., Hansen, J. E., Alexander, A. L., Chapman, E. J., Fahlgren, N., Allen, E. & Carrington, J. C. (2008). Specificity of ARGONAUTE7-miR390 interaction and dual functionality in TAS3 trans-acting siRNA formation. *Cell* **133**, 128-141.

Morel, J. B., Godon, C., Mourrain, P., Béclin, C., Boutet, S., Feuerbach, F., Proux, F. & Vaucheret, H. (2002). Fertile hypomorphic *ARGONAUTE* (*ago1*) mutants impaired in post- pranscriptional gene silencing and virus resistance. *Plant Cell* **14**, 629-639.

Moreno, I. M., Malpica, J. M., Díaz-Pendón, J. A., Moriones, E., Fraile, A. & García-Arenal, F. (2004). Variability and genetic structure of the population of watermelon mosaic virus infecting melon in Spain. *Virology* **318**, 451-460.

Mosher, R. A., Melnyk, C. W., Kelly, K. A., Dunn, R. M., Studholme, D. J. & Baulcombe, D. C. (2009). Uniparental expression of PolIV-dependent siRNAs in developing endosperm of Arabidopsis. *Nature* **460**, 283-U151.

Mourrain, P., Béclin, C., Elmayan, T., Feuerbach, F., Godon, C., Morel, J. B., Jouette, D., Lacombe, A. M., Nikic, S., Picault, N., Remoue, K., Sanial, M., Vo, T. A. & Vaucheret, H. (2000). *Arabidopsis SGS2* and *SGS3* genes are required for posttranscriptional gene silencing and natural virus resistance. *Cell* **101**, 533-542.

Moxon, S., Schwach, F., MacLean, D., Dalmay, T., Studholme, D. J. & Moulton, V. (2008). A toolkit for analysing large-scale plant small RNA datasets. *Bioinformatics*.

Mukasa, S. B., Rubaihayo, P. R. & Valkonen, J. P. T. (2006). Interactions between a crinivirus, an ipomovirus and a potyvirus in coinfected sweetpotato plants. *Plant Pathol* **55**, 458-467.

Nakagawa, T., Kurose, T., Hino, T., Tanaka, K., Kawamukai, M., Niwa, Y., Toyooka, K., Matsuoka, K., Jinbo, T. & Kimura, T. (2007). Development of series of gateway binary vectors, pGWBs, for realizing efficient construction of fusion genes for plant transformation. *J Biosci Bioeng* **104**, 34-41.

Napoli, C., Lemieux, C. & Jorgensen, R. (1990). Introduction of a chimeric chalcone synthase gene into petunia results in reversible co-suppression of homologous genes in trans. *Plant Cell* **2**, 279-289.

Nishiguchi, M., Yamasaki, S., Lu, X., Shimoyama, A., Hanada, K., Sonoda, S., Shimono, M., Sakai, J., Mikoshiba, Y. & Fujisawa, I. (2006). Konjak mosaic virus: the complete nucleotide sequence of the genomic RNA and its comparison with other potyviruses. *Arch Virol* **151**, 1643-1650.

Niu, Q.-W., Lin, S.-S., Reyes, J. L., Chen, K.-C., Wu, H.-W., Yeh, S.-D. & Chua, N.-H. (2006). Expression of artificial microRNAs in transgenic *Arabidopsis thaliana* confers virus resistance. *Nat Biotech* **24**, 1420-1428.

Nykänen, A., Haley, B. & Zamore, P. D. (2001). ATP requirements and small interfering RNA structure in the RNA interference pathway. *Cell* **107**, 309-321.

Omarov, R., Sparks, K., Smith, L., Zindovic, J. & Scholthof, H. B. (2006). Biological relevance of a stable biochemical interaction between the tombusvirus-encoded p19 and short interfering RNAs. *J Virol* **80**, 3000-3008.

Omarov, R. T., Ciomperlik, J. J. & Scholthof, H. B. (2007). RNAi-associated ssRNA-specific ribonucleases in *Tombusvirus* P19 mutant-infected plants and evidence for a discrete siRNA-containing effector complex. *Proc Natl Acad Sci USA* **104**, 1714-1719.

Otsuka, M., Jing, Q., Georgel, P., New, L., Chen, J., Mols, J., Kang, Y. J., Jiang, Z., Du, X., Cook, R., Das, S. C., Pattnaik, A. K., Beutler, B. & Han, J. (2007). Hypersusceptibility to vesicular stomatitis virus infection in Dicer1-deficient mice is due to impaired miR24 and miR93 expression. *Immunity* **27**, 123-134.

Pagan, I., Fraile, A., Fernandez-Fueyo, E., Montes, N., Alonso-Blanco, C. & Garcia-Arenal, F. (2010). Arabidopsis thaliana as a model for the study of plant-virus co-evolution. *Philos Trans R Soc Lond B Biol Sci* **365**, 1983-1995.

Pantaleo, V. & Burgyán, J. (2008). Cymbidium ringspot virus harnesses RNA silencing to control the accumulation of virus parasite satellite RNA. *J Virol* **82**, 11851-11858.

Pantaleo, V., Szittya, G. & Burgyan, J. (2007). Molecular bases of viral RNA targeting by viral small interfering RNA-programmed RISC. *J Virol* **81**, 3797-3806.

Park, W., Li, J., Song, R., Messing, J. & Chen, X. (2002). CARPEL FACTORY, a Dicer homolog, and HEN1, a novel Protein, act in microRNA metabolism in *Arabidopsis thaliana*. *Curr Biol* **12**, 1484-1495.

Payre, F., Buono, P., Vanzo, N. & Vincent, A. (1997). Two types of zinc fingers are required for dimerization of the serendipity delta transcriptional activator. *Mol Cell Biol* **17**, 3137-3145.

Pazhouhandeh, M., Dieterle, M., Marrocco, K., Lechner, E., Berry, B., Brault, V., Hemmer, O., Kretsch, T., Richards, K. E., Genschik, P. & Ziegler-Graff, V. (2006). F-box-like domain in the polerovirus protein P0 is required for silencing suppressor function. *Proc Natl Acad Sci USA* **103**, 1994-1999.

Pedersen, I. M., Cheng, G., Wieland, S., Volinia, S., Croce, C. M., Chisari, F. V. & David, M. (2007). Interferon modulation of cellular microRNAs as an antiviral mechanism. *Nature* **449**, 919-922.

Peng, Y. H., Kadoury, D., Gal-On, A., Wang, Y. & Raccah, B. (1998). Mutations in the HC-Pro gene of zucchini yellow mosaic potyvirus: effects on aphid transmission and binding to purified virions. *J Gen Virol* **79**, 897-904.

Petrzik, K. & Franova, J. (2006). Complete genome sequence of Daphne mosaic virus - a potyvirus from an ornamental shrub related to papaya leaf distortion mosaic virus. *Arch Virol* **151**, 1461-1465.

Plisson, C., Drucker, M., Blanc, S., German-Retana, S., Le Gall, O., Thomas, D. & Bron, P. (2003). Structural characterization of HC-Pro, a plant virus multifunctional protein. *J Biol Chem* **278**, 23753-23761.

Pooggin, M. & Hohn, T. (2003). RNAi targeting of DNA virus in plants. *Nat Biotech* **21**, 131-132.

Posada, D. (2002). Evaluation of methods for detecting recombination from DNA sequences: empirical data. *Molecular Biology and Evolution* **19**, 708-717.

Posada, D. & Crandall, K. A. (2002). The effect of recombination on the accuracy of phylogeny estimation. *J Mol Evol* **54**, 396-402.

Powers, J. G., Sit, T. L., Heinsohn, C., George, C. G., Kim, K. H. & Lommel, S. A. (2008a). The *Red clover necrotic mosaic virus* RNA-2 encoded movement protein is a second suppressor of RNA silencing. *Virology* **381**, 277-286.

Powers, J. G., Sit, T. L., Qu, F., Morris, T. J., Kim, K.-H. & Lommel, S. A. (2008b). A versatile assay for the identification of RNA silencing suppressors based on complementation of viral movement. *Mol Plant-Microbe Interact* **21**, 879-890.

Preall, J. B. & Sontheimer, E. J. (2005). RNAi: RISC gets loaded. *Cell* **123**, 543-545.

Pruss, G., Ge, X., Shi, X. M., Carrington, J. C. & Vance, V. B. (1997). Plant viral synergism: The potyviral genome encodes a broad-range pathogenicity enhancer that transactivates replication of heterologous viruses. *Plant Cell* **9**, 859-868.

Puustinen, P. & Mäkinen, K. (2004). Uridylylation of the potyvirus VPg by viral replicase NIb correlates with the nucleotide binding capacity of VPg. *J Biol Chem* **279**, 38103-38110.

Qi, Y., He, X., Wang, X. J., Kohany, O., Jurka, J. & Hannon, G. J. (2006). Distinct catalytic and non-catalytic roles of ARGONAUTE4 in RNA-directed DNA methylation. *Nature* **443**, 1008-1012.

Qiu, W. P., Park, J. W. & Scholthof, H. B. (2002). Tombusvirus p19-mediated suppression of virus-induced gene silencing is controlled by genetic and dosage features that influence pathogenicity. *Mol Plant-Microbe Interact* **15**, 269-280.

Qu, F., Ye, X. & Morris, T. J. (2008). *Arabidopsis* DRB4, AGO1, AGO7, and RDR6 participate in a DCL4-initiated antiviral RNA silencing pathway negatively regulated by DCL1. *Proc Natl Acad Sci USA* **105**, 14732-14737.

Qu, J., Ye, J. & Fang, R. (2007). Artificial microRNA-mediated virus resistance in plants. *J Virol* **81**, 6690-6699.

Rahim, M. D., Andika, I. B., Han, C., Kondo, H. & Tamada, T. (2007). RNA4-encoded p31 of beet necrotic yellow vein virus is involved in efficient vector transmission, symptom severity and silencing suppression in roots. *J Gen Virol* **88**, 1611-1619.

Raja, P., Sanville, B. C., Buchmann, R. C. & Bisaro, D. M. (2008). Viral genome methylation as an epigenetic defense against geminiviruses. *J Virol* **82**, 8997-9007.

Rajagopalan, R., Vaucheret, H., Trejo, J. & Bartel, D. P. (2006). A diverse and evolutionarily fluid set of microRNAs in Arabidopsis thaliana. *Genes Dev* **20**, 3407-3425.

Rajamäki, M.-L. & Valkonen, J. P. T. (1999). The 6K2 protein and the VPg of potato virus A are determinants of systemic infection in *Nicandra physaloides*. *Mol Plant-Microbe Interact* **12**, 1074-1081.

Rajamäki, M. L., Kelloniemi, J., Alminaite, A., Kekarainen, T., Rabenstein, F. & Valkonen, J. P. (2005). A novel insertion site inside the potyvirus P1 cistron allows expression of heterologous proteins and suggests some P1 functions. *Virology* **342**, 88-101.

Rajamaki, M. L. & Valkonen, J. P. T. (2009). Control of nuclear and nucleolar localization of nuclear inclusion protein a of picorna-like Potato virus A in Nicotiana species. *Plant Cell* **21**, 2485-2502.

Rakitina, D. V., Kantidze, O. L., Leshchiner, A. D., Solovyev, A. G., Novikov, V. K., Morozov, S. Y. & Kalinina, N. O. (2005). Coat proteins of two filamentous plant viruses display NTPase activity in vitro. *FEBS Lett* **579**, 4955-4960.

Rakitina, D. V., Yelina, N. E. & Kalinina, N. O. (2006). Zinc ions stimulate the cooperative RNA binding of hordeiviral γb protein. *FEBS Lett* **580**, 5077-5083.

Ramachandran, V. & Chen, X. (2008). Degradation of microRNAs by a family of exoribonucleases in Arabidopsis. *Science* **321**, 1490-1492.

Rand, T. A., Ginalski, K., Grishin, N. V. & Wang, X. (2004). Biochemical identification of Argonaute 2 as the sole protein required for RNA-induced silencing complex activity. *Proc Natl Acad Sci USA* **101**, 14385-14389.

Rand, T. A., Petersen, S., Du, F. & Wang, X. (2005). Argonaute2 cleaves the anti-guide strand of siRNA during RISC activation. *Cell* **123**, 621-629.

Ratcliff, F., Harrison, B. D. & Baulcombe, D. C. (1997). A similarity between viral defense and gene silencing in plants. *Science* **276**, 1558-1560.

Reinhart, B. J., Weinstein, E. G., Rhoades, M. W., Bartel, B. & Bartel, D. P. (2002). MicroRNAs in plants. *Genes Dev* **16**, 1616-1626.

Riechmann, J. L., Cervera, M. T. & García, J. A. (1995). Processing of the plum pox virus polyprotein at the P3-6K_1 junction is not required for virus viability. *J Gen Virol* **76**, 951-956.

Riechmann, J. L., Laín, S. & García, J. A. (1992). Highlights and prospects of potyvirus molecular biology. *J Gen Virol* **73**, 1-16.

Roberts, I. M., Wang, D., Findlay, K. & Maule, A. J. (1998). Ultrastructural and temporal observations of the potyvirus cylindrical inclusions (CIs) show that the CI protein acts transiently in aiding virus movement. *Virology* **245**, 173-181.

Rodríguez-Cerezo, E., Ammar, E., Pirone, E. D. & Shaw, J. G. (1993). Association of the non-structural P3 viral protein with cylindrical inclusions in potyvirus-infected cells. *J Gen Virol* **74**, 1945-1949.

Rodríguez-Cerezo, E., Findlay, K., Shaw, J. G., Lomonossoff, G. P., Qiu, S. G., Linstead, P., Shanks, M. & Risco, C. (1997). The coat and cylindrical inclusion proteins of a potyvirus are associated with connections between plant cells. *Virology* **236**, 296-306.

Rodríguez-Negrete, E. A., Carrillo-Tripp, J. & Rivera-Bustamante, R. F. (2009). RNA silencing against geminivirus: Complementary action of posttranscriptional gene silencing and transcriptional gene silencing in host recovery. *J Virol* **83**, 1332-1340.

Rohila, J. S., Chen, M., Cerny, R. & Fromm, M. E. (2004). Improved tandem affinity purification tag and methods for isolation of protein heterocomplexes from plants. *Plant J* **38**, 172-181.

Rojas, M. R., Zerbini, F. M., Allison, R. F., Gilbertson, R. L. & Lucas, W. J. (1997). Capsid protein and helper component proteinase function as potyvirus cell-to-cell movement proteins. *Virology* **237**, 283-295.

Roossinck, M. J. (2003). Plant RNA virus evolution. *Current Opinion in Microbiology* **6**, 406-409.

Roossinck, M. J. (2005). Symbiosis versus competition in plant virus evolution. *Nat Rev Microbiol* **3**, 917-924.

Ruby, J. G., Jan, C., Player, C., Axtell, M. J., Lee, W., Nusbaum, C., Ge, H. & Bartel, D. P. (2006). Large-scale sequencing reveals 21U-RNAs and additional microRNAs and endogenous siRNAs in C-elegans. *Cell* **127**, 1193-1207.

Ruiz-Ferrer, V., Boskovic, J., Alfonso, C., Rivas, G., Llorca, O., López-Abella, D. & López-Moya, J. J. (2005). Structural analysis of tobacco etch potyvirus HC-Pro oligomers involved in aphid transmission. *J Virol* **79**, 3758-3765.

Saito, K., Nishida, K. M., Mori, T., Kawamura, Y., Miyoshi, K., Nagami, T., Siomi, H. & Siomi, M. C. (2006). Specific association of Piwi with rasiRNAs derived from retrotransposon and heterochromatic regions in the *Drosophila* genome. *Genes Dev* **20**, 2214-2222.

Salvador, B., Saenz, P., Yanguez, E., Quiot, J. B., Quiot, L., Delgadillo, M. O., Garcia, J. A. & Simon-Mateo, C. (2008). Host-specific effect of P1 exchange between two potyviruses. *Mol Plant Pathol* **9**, 147-155.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular cloning: a laboratory manual*. Cold Spring Harbor, N.Y: Cold Spring Harbor Laboratory.

Saunders, L. R. & Barber, G. N. (2003). The dsRNA binding protein family: critical roles, diverse cellular functions. *FASEB J* **17**, 961-983.

Schaad, M. C., Haldeman-Cahill, R., Cronin, S. & Carrington, J. C. (1996). Analysis of the VPg-proteinase (NIa) encoded by tobacco etch potyvirus: Effects of mutations on subcellular transport, proteolytic processing, and genome amplification. *J Virol* **70**, 7039-7048.

Schaad, M. C., Jensen, P. E. & Carrington, J. C. (1997a). Formation of plant RNA virus replication complexes on membranes: role of an endoplasmic reticulum-targeted viral protein. *EMBO J* **16**, 4049-4059.

Schaad, M. C., Lellis, A. D. & Carrington, J. C. (1997b). VPg of tobacco etch potyvirus is a host genotype-specific determinant for long-distance movement. *J Virol* **71**, 8624-8631.

Schwab, R. & Voinnet, O. (2010). RNA silencing amplification in plants: Size matters. *Proc Natl Acad Sci U S A* **107**, 14945-14946.

Schwach, F., Vaistij, F. E., Jones, L. & Baulcombe, D. C. (2005). An RNA-dependent RNA polymerase prevents meristem invasion by Potato Virus X and is required for the activity but tot the production of a systemic silencing signal. *Plant Physiol* **138**, 1842-1852.

Schwartz, M., Chen, J. B., Janda, M., Sullivan, M., den Boon, J. & Ahlquist, P. (2002). A positive-strand RNA virus replication complex parallels form and function of retrovirus capsids. *Mol Cell* **9**, 505-514.

Schwarz, D. S., Hutvagner, G., Du, T., Xu, Z., Aronin, N. & Zamore, P. D. (2003). Asymmetry in the assembly of the RNAi enzyme complex. *Cell* **115**, 199-208.

Seemanpillai, M., Dry, I., Randles, J. & Rezaian, A. (2003). Transcriptional silencing of geminiviral promoter-driven transgenes following homologous virus infection. *Mol Plant-Microbe Interact* **16**, 429-438.

Shivaprasad, P. V., Rajeswaran, R., Blevins, T., Schoelz, J., Meins, F., Jr., Hohn, T. & Pooggin, M. M. (2008). The CaMV transactivator/viroplasmin interferes with RDR6-dependent *trans*-acting and secondary siRNA pathways in *Arabidopsis*. *Nucleic Acids Res* **36**, 5896-5909.

Shukla, D. D., Frenkel, M. J., McKern, N. M., Ward, C. W., Jilka, J., Tosic, M. & Ford, R. E. (1992). Present status of the sugarcane mosaic subgroup of potyviruses. *Arch Virol* **Suppl 5**, 363-373.

Shukla, D. D., Ward, C. W. & Brunt, A. A. (1994). *The Potyviridae*. Cambridge: CAB International.

Silhavy, D. & Burgyán, J. (2004). Effects and side-effects of viral RNA silencing suppressors on short RNAs. *Trends Plant Sci* **9**, 76-83.

Simón-Buela, L., Guo, H. S. & García, J. A. (1997). Cap-independent leaky scanning as the mechanism of translation initiation of a plant viral genomic RNA. *J Gen Virol* **78**, 2691-2699.

Simón-Mateo, C. & García, J. A. (2006). MicroRNA-guided processing impairs Plum pox virus replication, but the virus readily evolves to escape this silencing mechanism. *J Virol* **80**, 2429-2436.

Smith, L. M., Pontes, O., Searle, I., Yelina, N., Yousafzai, F. K., Herr, A. J., Pikaard, C. S. & Baulcombe, D. C. (2007). An SNF2 protein associated with nuclear RNA silencing and the spread of a silencing signal between cells in *Arabidopsis*. *Plant Cell* **19**, 1507-1521.

Soards, A. J., Murphy, A. M., Palukaltis, P. & Carr, J. P. (2002). Virulence and differential local and systemic spread of Cucumber mosaic virus in tobacco are affected by the CMV 2b protein. *Molecular Plant Microbe Interactions* **15**, 647-653.

Souret, F. F., Kastenmayer, J. P. & Green, P. J. (2004). AtXRN4 degrades mRNA in *Arabidopsis* and its substrates include selected miRNA targets. *Mol Cell* **15**, 173-183.

Spetz, C., Taboada, A. M., Darwich, S., Ramsell, J., Salazar, L. F. & Valkonen, J. P. T. (2003). Molecular resolution of a complex of potyviruses infecting solanaceous crops at the centre of origin in Peru. *Journal of General Virology* **84**, 2565-2578.

Spetz, C. & Valkonen, J. P. T. (2004). Potyviral 6K2 protein long-distance movement and symptom-induction functions are independent and host-specific. *Mol Plant-Microbe Interact* **17**, 502-510.

Steiner, F. A., Hoogstrate, S. W., Okihara, K. L., Thijssen, K. L., Ketting, R. F., Plasterk, R. H. A. & Sijen, T. (2007). Structural features of small RNA precursors determine Argonaute loading in Caenorhabditis elegans. *Nat Struct Mol Biol* **14**, 927-933.

Stenger, D. C., Hein, G. L. & French, R. (2006). Nested deletion analysis of *Wheat streak mosaic virus* HC-Pro: Mapping of domains affecting polyprotein processing and eriophyid mite transmission. *Virology* **350**, 465-474.

Stenger, D. C., Hein, G. L., Gildow, F. E., Horken, K. M. & French, R. (2005). Plant virus HC-Pro is a determinant of eriophyid mite transmission. *J Virol* **79**, 9054-9061.

Stenger, D. C., Young, B. A., Qu, F., Morris, T. J. & French, R. (2007). *Wheat streak mosaic virus* lacking helper component-proteinase is competent to produce disease synergism in double infections with *Maize chlorotic mottle virus*. *Phytopathology* **97**, 1213-1221.

Stenger, D. C., Young, B.A., Qu, F., Morris, T., French, R.C. (2007). Wheat streak mosaic virus P1, not HC-Pro, facilitates disease synergism and suppression of post-transcriptional gene silencing. *Phytopathology 97:S111*.

Sullivan, C. S. & Ganem, D. (2005a). A virus-encoded inhibitor that blocks RNA interference in mammalian cells. *J Virol* **79**, 7371-7379.

Sullivan, C. S. & Ganem, D. (2005b). MicroRNAs and viral infection. *Mol Cell* **20**, 3-7.

Sunter, G. & Bisaro, D. M. (1992). Transactivation of geminivirus AR1 and BR1 gene expression by the viral AL2 gene product occurs at the level of transcription. *Plant Cell* **4**, 1321-1331.

Susi, P., Hohkuri, M., Wahlroos, T. & Kilby, N. J. (2004). Characteristics of RNA silencing in plants: similarities and differences across kingdoms. *Plant Mol Biol* **54**, 157-174.

Szittya, G., Molnar, A., Silhavy, D., Hornyik, C. & Burgyán, J. (2002). Short defective interfering RNAs of tombusviruses are not targeted but trigger post-transcriptional gene silencing against their helper virus. *Plant Cell* **14**, 359-372.

Takeda, A., Iwasaki, S., Watanabe, T., Utsumi, M. & Watanabe, Y. (2008). The mechanism selecting the guide strand from small RNA duplexes is different among Argonaute proteins. *Plant Cell Physiol* **49**, 493-500.

Takeda, A., Sugiyama, K., Nagano, H., Mori, M., Kaido, M., Mise, K., Tsuda, S. & Okuno, T. (2002). Identification of a novel RNA silencing suppressor, NSs protein of *Tomato spotted wilt virus*. *FEBS Lett* **532**, 75-79.

Takeda, A., Tsukuda, M., Mizumoto, H., Okamoto, K., Kaido, M., Mise, K. & Okuno, T. (2005). A plant RNA virus suppresses RNA silencing through viral RNA replication. *EMBO J* **24**, 3147-3157.

Taliansky, M., Roberts, I. M., Kalinina, N., Ryabov, E. V., Raj, S. K., Robinson, D. J. & Oparka, K. J. (2003). An umbraviral protein, involved in long-distance RNA movement, binds viral RNA and forms unique, protective ribonucleoprotein complexes. *J Virol* **77**, 3031-3040.

Tan, Z., Wada, Y., Chen, J. & Ohshima, K. (2004). Inter- and intralineage recombinants are common in natural populations of *Turnip mosaic virus*. *Journal of General Virology* **85**, 2683-2696.

Till, S., Lejeune, E., Thermann, R., Bortfeld, M., Hothorn, M., Enderle, D., Heinrich, C., Hentze, M. W. & Ladurner, A. G. (2007). A conserved motif in Argonaute-interacting proteins mediates functional interactions through the Argonaute PIWI domain. *Nat Struct Mol Biol* **14**, 897-903.

Tolia, N. H. & Joshua-Tor, L. (2007). Slicer and the Argonautes. *Nat Chem Biol* **3**, 36-43.

Tomari, Y., Du, T. & Zamore, P. D. (2007). Sorting of *Drosophila* small silencing RNAs. *Cell* **130**, 299-308.

Tomari, Y., Du, T. T., Haley, B., Schwarz, D. S., Bennett, R., Cook, H. A., Koppetsch, B. S., Theurkauf, W. E. & Zamore, P. D. (2004). RISC assembly defects in the Drosophila RNAi mutant armitage. *Cell* **116**, 831-841.

Triboulet, R., Mari, B., Lin, Y. L., Chable-Bessia, C., Bennasser, Y., Lebrigand, K., Cardinaud, B., Maurin, T., Barbry, P., Baillat, V., Reynes, J., Corbeau, P., Jeang, K. T. & Benkirane, M. (2007). Suppression of microRNA-silencing pathway by HIV-1 during virus replication. *Science* **315**, 1579-1582.

Trinks, D., Rajeswaran, R., Shivaprasad, P. V., Akbergenov, R., Oakeley, E. J., Veluthambi, K., Hohn, T. & Pooggin, M. M. (2005). Suppression of RNA silencing by a geminivirus nuclear protein, AC2, correlates with transactivation of host genes. *J Virol* **79**, 2517-2527.

Urcuqui-Inchima, S., Haenni, A. L. & Bernardi, F. (2001). Potyvirus proteins: a wealth of functions. *Virus Res* **74**, 157-175.

Urcuqui-Inchima, S., Maya, I. G., Drugeon, G., Haenni, A. L. & Bernardi, F. (1999). Effect of mutations within the Cys-rich motif of potyvirus helper component-proteinase on self-interaction. *J Gen Virol* **80**, 2809-2812.

Valli, A., López-Moya, J. J. & García, J. A. (2009). RNA silencing and its suppressors in the plant-virus interplay. In *Encyclopedia of Life Sciences (ELS)*, p. http://www.els.net/ [DOI: 10.1002/9780470015902.a9780470021261]. Chichester: John Wiley & Sons, Ltd.

van der Krol, A. R., Mur, L. A., Beld, M., Mol, J. N. & Stuitje, A. R. (1990). Flavonoid genes in petunia: addition of a limited number of gene copies may lead to a suppression of gene expression. *Plant Cell* **2**, 291-299.

van Rij, R. P., Saleh, M. C., Berry, B., Foo, C., Houk, A., Antoniewski, C. & Andino, R. (2006). The RNA silencing endonuclease Argonaute 2 mediates specific antiviral immunity in Drosophila melanogaster. *Genes Dev* **20**, 2985-2995.

van Wezel, R., Dong, X. L., Liu, H. T., Tien, P., Stanley, J. & Hong, Y. G. (2002). Mutation of three cysteine residues in Tomato yellow leaf curl virus-China C2 protein causes dysfunction in pathogenesis and posttranscriptional gene-silencing suppression. *Mol Plant-Microbe Interact* **15**, 203-208.

Vanderschuren, H., Akbergenov, R., Pooggin, M. M., Hohn, T., Gruissem, W. & Zhang, P. (2007). Transgenic cassava resistance to African cassava mosaic virus is enhanced by viral DNA-A bidirectional promoter-derived siRNAs. *Plant Mol Biol* **64**, 549-557.

Vanitharani, R., Chellappan, P., Pita, J. S. & Fauquet, C. M. (2004). Differential roles of AC2 and AC4 of cassava geminiviruses in mediating synergism and suppression of posttranscriptional gene silencing. *J Virol* **78**, 9487-9498.

Vargason, J. M., Szittya, G., Burgyan, J. & Tanaka Hall, T. M. (2003). Size selective recognition of siRNA by an RNA silencing suppressor. *Cell* **115**, 799-811.

Vasudevan, S., Tong, Y. & Steitz, J. A. (2007). Switching from repression to activation: microRNAs can up-regulate translation. *Science* **318**, 1931-1934.

Vaucheret, H. (2006). Post-transcriptional small RNA pathways in plants: mechanisms and regulations. *Genes Dev* **20**, 759-771.

Vaucheret, H. (2008). Plant ARGONAUTES. *Trends Plant Sci* **13**, 350-358.

Vaucheret, H., Vazquez, F., Crete, P. & Bartel, D. P. (2004). The action of ARGONAUTE1 in the miRNA pathway and its regulation by the miRNA pathway are crucial for plant development. *Genes Dev* **18**, 1187-1197.

Vazquez, F., Gasciolli, V., Crete, P. & Vaucheret, H. (2004). The nuclear dsRNA binding protein HYL1 Is required for microRNA accumulation and plant development, but not posttranscriptional transgene silencing. *Curr Biol* **14**, 346-351.

Verchot, J. & Carrington, J. C. (1995). Evidence that the potyvirus P1 proteinase functions in trans as an accessory factor for genome amplification. *J Virol* **69**, 3668-3674.

Verchot, J., Herndon, K. L. & Carrington, J. C. (1992). Mutational analysis of the tobacco etch potyviral 35-kDa proteinase: Identification of essential residues and requirements for autoproteolysis. *Virology* **190**, 298-306.

Verchot, J., Koonin, E. V. & Carrington, J. C. (1991). The 35-kDa protein from the N-terminus of a potyviral polyprotein functions as a third virus-encoded proteinase. *Virology* **185**, 527-535.

Verdel, A., Jia, S., Gerber, S., Sugiyama, T., Gygi, S., Grewal, S. I. & Moazed, D. (2004). RNAi-mediated targeting of heterochromatin by the RITS complex. *Science* **303**, 672-676.

Vogler, H., Akbergenov, R., Shivaprasad, P. V., Dang, V., Fasler, M., Kwon, M. O., Zhanybekova, S., Hohn, T. & Heinlein, M. (2007). Modification of small RNAs associated with suppression of RNA silencing by tobamovirus replicase protein. *J Virol* **81**, 10379-10288.

Voinnet, O. (2001). RNA silencing as a plant immune system against viruses. *Trends Genet* **17**, 449-459.

Voinnet, O. (2005). Induction and suppression of RNA silencing: insights from viral infections. *Nat Rev Genet* **6**, 206-220.

Voinnet, O. & Baulcombe, D. C. (1997). Systemic signalling in gene silencing. *Nature* **389**, 553.

Voinnet, O., Pinto, Y. M. & Baulcombe, D. C. (1999). Suppression of gene silencing: A general strategy used by diverse DNA and RNA viruses of plants. *Proc Natl Acad Sci USA* **96**, 14147-14152.

Voinnet, O., Rivas, S., Mestre, P. & Baulcombe, D. (2003). An enhanced transient expression system in plants based on suppression of gene silencing by the p19 protein of tomato bushy stunt virus. *Plant J* **33**, 949-956.

Waltermann, A. & Maiss, E. (2006). Detection of 6K1 as a mature protein of 6 kDa in plum pox virus-infected *Nicotiana benthamiana*. *J Gen Virol* **87**, 2381-2386.

Wang, H., Buckley, K. J., Yang, X. J., Buchmann, R. C. & Bisaro, D. M. (2005). Adenosine kinase inhibition and suppression of RNA silencing by geminivirus AL2 and L2 proteins. *J Virol* **79**, 7410-7418.

Wang, H., Hao, L., Shung, C. Y., Sunter, G. & Bisaro, D. M. (2003). Adenosine kinase is inactivated by geminivirus AL2 and L2 proteins. *Plant Cell* **15**, 3020-3032.

Wang, M. B., Bian, X. Y., Wu, L. M., Liu, L. X., Smith, N. A., Isenegger, D., Wu, R. M., Masuta, C., Vance, V. B., Watson, J. M., Rezaian, A., Dennis, E. S. & Waterhouse, P. M. (2004). On the role of RNA silencing in the pathogenicity and evolution of viroids and viral satellites. *Proc Natl Acad Sci USA* **101**, 3275-3280.

Wang, R. Y., Powell, G., Hardie, J. & Pirone, T. P. (1998). Role of the helper component in vector-specific transmission of potyviruses. *J Gen Virol* **79**, 1519-1524.

Wang, X., Ullah, Z. & Grumet, R. (2000). Interaction between Zucchini Yellow Mosaic Potyvirus RNA-Dependent RNA Polymerase and Host Poly-(A) Binding Protein. *Virology* **275**, 433-443.

Wang, X. B., Wu, Q., Ito, T., Cillo, F., Li, W. X., Chen, X., Yu, J. L. & Ding, S. W. (2010). RNAi-mediated viral immunity requires amplification of virus-derived siRNAs in Arabidopsis thaliana. *Proc Natl Acad Sci USA* **107**, 484-489.

Ward, C. W. & Shukla, D. D. (1991). Taxonomy of potyviruses: Current problems and some solutions. *Intervirology* **32**, 269-296.

Wei, T., Zhang, C., Hong, J., Xiong, R., Kasschau, K. D., Zhou, X., Carrington, J. C. & Wang, A. (2010a). Formation of complexes at plasmodesmata for potyvirus intercellular movement is mediated by the viral protein P3N-PIPO. *PLoS Pathog* **6**, e1000962.

Wei, T. Y., Huang, T. S., McNeil, J., Laliberte, J. F., Hong, J., Nelson, R. S. & Wang, A. M. (2010b). Sequential recruitment of the endoplasmic reticulum and chloroplasts for plant potyvirus replication. *J Virol* **84**, 799-809.

Wen, R. H. & Hajimorad, M. R. (2010). Mutational analysis of the putative pipo of soybean mosaic virus suggests disruption of PIPO protein impedes movement. *Virology* **400**, 1-7.

Xie, Z., Allen, E., Wilken, A. & Carrington, J. C. (2005). DICER-LIKE 4 functions in trans-acting small interfering RNA biogenesis and vegetative phase change in *Arabidopsis thaliana*. *Proc Natl Acad Sci USA* **102**, 12984-12989.

Xie, Z., Johansen, L. K., Gustafson, A. M., Kasschau, K. D., Lellis, A. D., Zilberman, D., Jacobsen, S. E. & Carrington, J. C. (2004). Genetic and functional diversification of small RNA pathways in plants. *PLoS Biology* **2**, 1-11.

Yaegashi, H., Takahashi, T., Isogai, M., Kobori, T., Ohki, S. & Yoshikawa, N. (2007a). Apple chlorotic leaf spot virus 50 kDa movement protein acts as a suppressor of systemic silencing without interfering with local silencing in *Nicotiana benthamiana*. *J Gen Virol* **88**, 316-324.

Yaegashi, H., Tamura, A., Isogai, M. & Yoshikawa, N. (2008). Inhibition of long-distance movement of RNA silencing signals in *Nicotiona benthamiana* by *Apple chlorotic leaf spot virus* 50 kDa movement protein. *Virology* **382**, 199-206.

Yaegashi, H., Yamatsuta, T., Takahashi, T., Li, C., Isogai, M., Kobori, T., Ohki, S. & Yoshikawa, N. (2007b). Characterization of virus-induced gene silencing in tobacco plants infected with apple latent spherical virus. *Arch Virol* **152**, 1839-1849.

Yang, L., Liu, Z., Lu, F., Dong, A. & Huang, H. (2006). SERRATE is a novel nuclear regulator in primary microRNA processing in Arabidopsis. *Plant J* **47**, 841-850.

Ye, K., Malinina, L. & Patel, D. J. (2003). Recognition of small interfering RNA by a viral suppressor of RNA silencing. *Nature* **426**, 874-878.

Ye, K. Q. & Patel, D. J. (2005). RNA silencing suppressor p21 of beet yellows virus forms an RNA binding octameric ring structure. *Structure* **13**, 1375-1384.

Yoshikawa, M., Peragine, A., Park, M. Y. & Poethig, R. S. (2005). A pathway for the biogenesis of trans-acting siRNAs in Arabidopsis. *Genes Dev* **19**, 2164-2175.

Yu, B., Bi, L., Zheng, B., Ji, L., Chevalier, D., Agarwal, M., Ramachandran, V., Li, W., Lagrange, T., Walker, J. C. & Chen, X. (2008). The FHA domain proteins DAWDLE in Arabidopsis and SNIP1 in humans act in small RNA biogenesis. *Proc Natl Acad Sci USA* **105**, 10073-10078.

Yu, B., Chapman, E. J., Yang, Z., Carrington, J. C. & Chen, X. (2006). Transgenically expressed viral RNA silencing suppressors interfere with microRNA methylation in Arabidopsis. *FEBS Lett* **580**, 3117-3120.

Yu, B., Yang, Z. Y., Li, J. J., Minakhina, S., Yang, M. C., Padgett, R. W., Steward, R. & Chen, X. M. (2005). Methylation as a crucial step in plant microRNA biogenesis. *Science* **307**, 932-935.

Zeenko, V. & Gallie, D. R. (2005). Cap-independent translation of tobacco etch virus is conferred by an RNA pseudoknot in the 5 '-leader. *J Biol Chem* **280**, 26813-26824.

Zhang, X., Du, P., Lu, L., Xiao, Q., Wang, W., Cao, X., Ren, B., Wei, C. & Li, Y. (2008). Contrasting effects of HC-Pro and 2b viral suppressors from *Sugarcane mosaic virus* and *Tomato aspermy cucumovirus* on the accumulation of siRNAs. *Virology* **374**, 351-360.

Zhang, X., Yuan, Y.-R., Pei, Y., Lin, S.-S., Tuschl, T., Patel, D. J. & Chua, N.-H. (2006). *Cucumber mosaic virus*-encoded 2b suppressor inhibits *Arabidopsis* Argonaute1 cleavage activity to counter plant defense. *Genes Dev* **20**, 3255-3268.

Zheng, X., Zhu, J., Kapoor, A. & Zhu, J. K. (2007). Role of *Arabidopsis* AGO6 in siRNA accumulation, DNA methylation and transcriptional gene silencing. *EMBO J* **26**, 1691-1701.

Zhong, Y., Guo, A., Li, C., Zhuang, B., Lai, M., Wei, C., Luo, J. & Li, Y. (2005). Identification of a naturally occurring recombinant isolate of *Sugarcane mosaic virus* causing maize dwarf mosaic disease. *Virus Genes* **30**, 75-83.

Zhou, Z., Dell'orco, M., Saldarelli, P., Turturo, C., Minafra, A. & Martelli, G. P. (2006). Identification of an RNA-silencing suppressor in the genome of *Grapevine virus A*. *J Gen Virol* **87**, 2387-2395.

Ziebell, H. & Carr, J. P. (2009). Effects of dicer-like endoribonucleases 2 and 4 on infection of Arabidopsis thaliana by cucumber mosaic virus and a mutant virus lacking the 2b counter-defence protein gene. *J Gen Virol* **90**, 2288-2292.

Ziebell, H., Payne, T., Berry, J. O., Walsh, J. A. & Carr, J. P. (2007). A cucumber mosaic virus mutant lacking the 2b counter-defence protein gene provides protection against wild-type strains. *Journal of General Virology* **88**, 2862-2871.

Zilberman, D., Cao, X. & Jacobsen, S. E. (2003). *ARGONAUTE4* control of locus-specific siRNA accumulation and DNA and histone methylation. *Science* **299**, 716-719.

Zrachya, A., Glick, E., Levy, Y., Arazi, T., Citovsky, V. & Gafni, Y. (2007). Suppressor of RNA silencing encoded by *Tomato yellow leaf curl virus*-Israel. *Virology* **358**, 159-165.